Renin-angiotensin system in intestinal inflammation – experimental studies with therapeutic interventions

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ACADEMIC DISSERTATION

To be presented, with the permission of Faculty of Medicine of the University of Helsinki, for public examination in Lecture Hall 2, Haartman Institute, Haartmaninkatu 3, on 15th of November 2019 at 12 noon.

HELSINKI 2019

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ISBN 978-951-51-5512-2 (print)
ISBN 978-951-51-5513-9 (PDF)
http://ethesis.helsinki.fi/
Unigrafia Oy 2019

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“Doubt kills more dreams than failure ever will.”

-Suzy Kassem

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I. **Salmenkari H**, Issakainen T, Vapaatalo H, Korpela R. 2015. Local corticosterone production and angiotensin-I converting enzyme shedding in a mouse model of intestinal inflammation. World J Gastroenterol. 21: 10072–10079.

II. **Salmenkari H**, Holappa M, Forsgård RA, Korpela R, Vapaatalo H. 2017. Orally administered angiotensin-converting enzyme-inhibitors captopril and isoleucine-proline-proline have distinct effects on local renin-angiotensin system and corticosterone synthesis in dextran sulfate sodium-induced colitis in mice. J Physiol Pharmacol. 68:355-362.

III. **Salmenkari H**, Pasanen L, Lindén J, Korpela R, Vapaatalo H. 2018. Beneficial anti-inflammatory effect of angiotensin-converting enzyme inhibitor and angiotensin receptor blocker in the treatment of dextran sulfate sodium-induced colitis in mice. J Physiol Pharmacol. 69. Epub 2018 Nov 7.

IV. **Salmenkari H**, Laitinen A, Forsgård RA, Holappa M, Lindén J, Pasanen L, Korhonen M, Korpela R, Nystedt J. 2019. The use of unlicensed bone marrow–derived platelet lysate–expanded mesenchymal stromal cells in colitis: a pre-clinical study. Cytotherapy. 21:175-188.

ABBREVIATIONS

ACE	Angiotensin-converting enzyme
ACTH	Adrenocorticotropic hormone
ADAM9	A disintegrin and a metalloprotease 9
ANG I	Angiotensin I
ANG II	Angiotensin II
AT1R	Angiotensin II receptor 1
AT2R	Angiotensin II receptor 2
cAMP	Cyclic adenosine monophosphate
CCL	CC chemokine ligand
cDNA	Complementary DNA
CYP	Cytochrome P450 enzyme
DAI	Disease activity index
DSS	Dextran sodium sulfate
ER	Endoplasmic reticulum
IBD	Inflammatory bowel disease
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon-γ
IL	Interleukin
LRH-1	Liver receptor homolog-1
MAdCAM-1	Mucosal addressin cell adhesion molecule-1
M cell	Microfold cell
mRNA	Messenger RNA
MSC	Mesenchymal stromal cell
NF-κB	Nuclear factor-kappa B
PGE2	Prostaglandin E2
PPARγ	Peroxisome proliferator activated receptor gamma
RAS	Renin-angiotensin system
RT-qPCR	Quantitative reverse transcription polymerase chain reaction

TGF-β	Transforming growth factor-β
Th cell	T helper cell
TNBS	2,4,6-trinitrobenzene sulfonic acid
TNFα	Tumor necrosis factor-α

ABSTRACT

The intestine is a major site of immune activity, and disturbances in the balance of proinflammatory and anti-inflammatory signals can lead to difficult and chronic diseases, like inflammatory bowel diseases, manifesting in uncontrolled inflammation in intestine. Local intestinal renin-angiotensin system (RAS) and glucocorticoid synthesis are recently uncovered complex mechanisms participating in the pathophysiology of intestinal diseases. These systems can offer new therapy options, either by repurposing well-known drugs like angiotensin-converting enzyme (ACE) inhibitors and angiotensin II receptor blockers or by novel innovations like mesenchymal stromal cell therapy.

The aim of this thesis was to investigate intestinal RAS and glucocorticoid production in intestinal inflammation and examine potential treatments with the focus on these two systems. RAS and glucocorticoid production and their possible interactions in intestine were characterized in a dextran sodium sulfate (DSS)-induced experimental colitis model using *in vitro* stimulations and inhibition of RAS *in vivo*. Glucocorticoid synthesis and ACE shedding were investigated as the release of corticosterone and ACE protein from live tissue to incubation media *in vitro*. The effects of ACE inhibitor, captopril, and ACE-inhibiting milk-derived bioactive tripeptide, Ile-Pro-Pro, were examined on intestinal RAS and glucocorticoid synthesis. ACE inhibitor, enalapril, and angiotensin II receptor blocker, losartan, were tested alone and in combination in alleviation of colitis. Finally, freshly cultivated and cryopreserved platelet-lysate expanded mesenchymal stromal cells were compared and their feasibility was examined in the treatment of colitis.

Enalapril and losartan were effective at alleviating colitis and lessening inflammation on their own but were without synergistic effects, supporting their potential to be investigated in clinical trials. MSC treatments proved feasible and without adverse effects, and freshly cultivated MSC treatments

had a modest anti-inflammatory effect reflected by reduction in proinflammatory cytokine levels. We found a specific induction of ACE ectodomain shedding in distal colon, the most affected region in DSS-induced colitis, and in proximal colon following a high DSS dose. ACE shedding could be downregulated by cryopreserved MSCs and an ACE-inhibiting tripeptide Ile-Pro-Pro treatment *in vivo*. These data imply that cell-surface ACE levels are actively regulated in intestinal inflammation, which could be a feedback mechanism to reduce the proinflammatory angiotensin II (Ang II) signaling. Ang II induced glucocorticoid production in small intestine incubations *in vitro*, thus implying of an anti-inflammatory property in Ang II signaling. We suggest that Ang II enhances the TNFα-mediated induction of glucocorticoid production during intestinal inflammation. *In vitro* or *in vivo* inhibition of Ang II production or signaling did not modulate intestinal glucocorticoid production, although captopril abolished the gene expression of the rate-limiting enzyme of glucocorticoid synthesis, *Cyp11b1*, *in vivo*.

1 INTRODUCTION

Inflammation is a vital process to protect organisms from pathogens and injuries. It is orchestrated by the immune system, which consists of highly specified organs and cells, leukocytes, which function in concert with the host tissues to maintain homeostasis between self and the outside world. Majority of this contact takes place in the intestine, where immune system must discriminate appropriately between dietary antigens, commensal bacteria and pathogens. Failure to control inflammation contributes to the pathogenesis of plethora of disorders and diseases.

Inflammatory bowel diseases (IBD) cause chronic inflammation of the intestine. They are a major disease burden in the Western world and their prevalence is increasing in the developing countries together with changing lifestyle (Ng et al., 2018). The processes leading to aberrant inflammation of the intestine in IBD are complex and treatments target them with anti-inflammatory and immunomodulatory drugs, and biologics to block cytokine signaling or leukocyte homing and adhesion. Many of the current therapies cause difficult adverse effects and drug resistance, raising a need for new treatment options.

Renin-angiotensin system (RAS) is a critical regulator of blood pressure, but in addition it participates in several physiological and pathophysiological functions locally in intestine including inflammation, fibrosis and apoptosis (Fyhrquist and Saijonmaa, 2008). RAS activity can be targeted with ACE inhibitors and angiotensin II receptor blockers, which have been efficient at treating colitis in preclinical studies (Jahovic et al., 2005). Mesenchymal stromal cell therapy is a new treatment option for IBD, but the cell origin and culture conditions affect their properties and potency (Gregoire et al., 2017), prompting thorough evaluation of feasible manufacturing strategies. Glucocorticoids are anti-inflammatory steroid hormones mainly produced by the adrenal cortex, but also locally in intestine, where they participate in

maintaining immune homeostasis (Cima et al., 2004). Inflammation in the intestine stimulates local corticosterone production, but there are disturbances in the process in IBD patients (Coste et al., 2007).

The studies in this thesis focused on RAS in intestinal inflammation. As RAS and glucocorticoids participate in opposite processes of inflammation, we sought to elucidate the possible interactions of RAS and glucocorticoids and investigate them in a setting of intestinal inflammation. We tested treatments for intestinal inflammation with commonly used antihypertensive drugs inhibiting RAS at different levels, and cell therapy using mesenchymal stromal cells, in a dextran sodium sulfate mouse model of colitis.

2 REVIEW OF THE LITERATURE

2.1 Intestine and inflammation

2.1.1 Intestinal homeostasis

Intestine is responsible for fluid and nutrient absorption, but also functions as a barrier between the organism and its environment, and thus is a major site of immune system. Intestinal immune system is in constant contact with dietary and microbial antigens, which results in a permanent state of benign immune activity in the intestine (MacDonald et al., 2011). This constitutive immune activation is termed immune homeostasis, and its purpose is to maintain tolerance to commensal bacteria and avoid overexaggerated immune responses while simultaneously preventing microbial overgrowth (Abraham and Medzhitov, 2011; MacDonald et al., 2011). Immune system has several ways to detect luminal antigens and train immune cells to maintain homeostasis, which is vital for the wellbeing of the organism, as disruption of homeostasis is thought to be one factor facilitating destructive chronic inflammation (Okumura and Takeda, 2017).

Intestinal epithelium (Figure 1) and mucus form the first line of defense against luminal microbes, antigens and toxic agents. Epithelial cells form at the base of intestinal crypts and migrate towards surface of the lumen and create a rapidly renewing physical barrier due to their tight junctions, which prevent luminal antigens and microbes from entering the mucosa (Goll and van Beelen Granlund, 2015). Goblet cells of the epithelium produce a protecting mucus layer which limits epithelial contact to harmful microbes (Johansson et al., 2011). Most of the epithelial cells are enterocytes which participate in sensing of luminal and mucosal antigens, antigen delivery to antigen-presenting cells and mediation of tolerance to commensal bacteria (Goll and van Beelen Granlund, 2015; MacDonald et al., 2011; Snoeck et al., 2005). Enteroendocrine cells sense luminal composition, relay signals by

secreting hormones and regulate the function of other cells (Garrett et al., 2010). Paneth cells and microfold cells (M cells) reside mainly in the small intestine and participate in luminal antigen sensing. Paneth cells produce antimicrobial agents and M cells deliver antigens to antigen-presenting cells. Intraepithelial lymphocytes reside between epithelial cells. (Garrett et al., 2010; Goll and van Beelen Granlund, 2015; MacDonald et al., 2011)

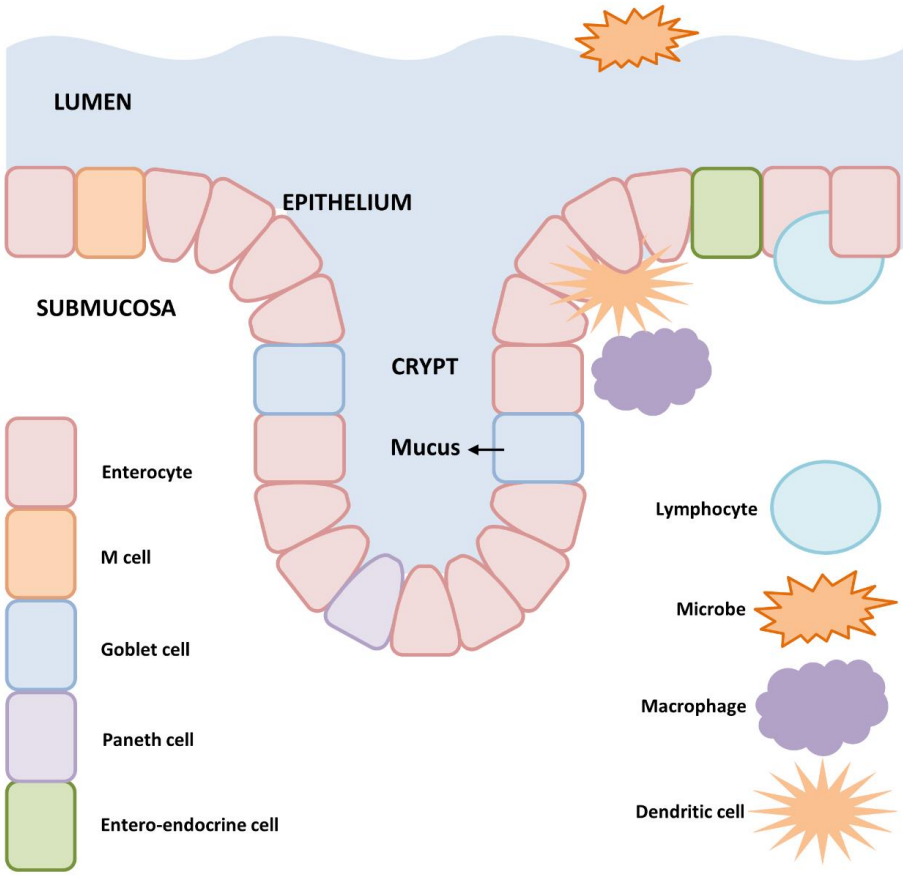

Figure 1. Composition of the cell types in intestinal epithelium that contribute to immune homeostasis. Modified from Okumura and Takeda, 2017.

Sensing of microbial antigens in epithelial and immune cells is mediated by pattern-recognition receptors, like Toll-like receptors and nucleotide-binding oligomerization domain-containing proteins. They reside on the cellular membrane or lysosomes of phagocytotic cells, and mediate either anti-inflammatory or proinflammatory responses depending on receptor location and intensity of the signal. In normal conditions, epithelial and antigen-presenting cells secrete anti-inflammatory and immunosuppressive transforming growth factor-β (TGF-β), prostaglandin E2 (PGE2) and interleukin(IL)-10 to maintain tolerance to commensal bacteria and downregulate inflammatory processes. (Garrett et al., 2010; Goll and van Beelen Granlund, 2015; MacDonald et al., 2011)

Immune cells are abundant both in epithelium and *lamina propria*. Intraepithelial lymphocytes reside between epithelial cells and participate in maintaining epithelial barrier and immune homeostasis. *Lamina propria* contains a large population of leukocytes, namely macrophages, dendritic cells and T cells. Dendritic cells can penetrate the epithelium to identify luminal antigens and present them to T cells in Peyer's patches or mesenteric lymph nodes, which are specialized lymphoid tissues of the intestine, and thus maintain tolerance to commensal bacteria. The resident macrophages and T cells display only partial capacity for activation by inflammatory stimuli. Resident intestinal macrophages phagocytose microbes and their products, and apoptotic epithelial cells, thus clearing excess *lamina propria* antigens, and produce anti-inflammatory cytokines to regulate other immune cells (Denning et al., 2007), and stimulate renewal of epithelium by secreting PGE2 (Bain and Mowat, 2014). A population of anti-inflammatory resident T cells display similar reduced activation of T-cell signaling via T-cell receptor and suppress immune activation. (Garrett et al., 2010; MacDonald et al., 2011)

2.1.2 Intestinal inflammation

Inflammation is an inherent process to remove pathogenic microbes, toxins, and damaged cells and enable wound repair. Acute inflammation occurs at the immediate appearance of tissue damage or pathogens as resident immune cells release proinflammatory mediators to facilitate vasodilation, increased vascular permeability, extravasation of leukocytes, mainly neutrophils and macrophages, and blood coagulation. The resulting influx of plasma and immune cells to the affected tissue leads to edema, intensifying of proinflammatory signals and phagocytosis of pathogens and cell debris, increased sensation of pain, and activation of complement system if pathogens are present. When the initial inflammatory stimulus is cleared, the proinflammatory signaling ceases and anti-inflammatory mediators are produced, and the inflammation resolves. Inability to clear the initial stimulus, or dysregulation of inflammation and its resolution leads to chronic inflammation manifesting in tissue damage, hypoxia, and fibrosis. Acute and chronic inflammation are distinctive immune cell and cytokine profiles. (Gilroy and De Maeyer, 2015)

In the intestine, disruption of the epithelial barrier and the subsequent translocation of bacteria or their antigens into the intestinal mucosa trigger inflammatory processes (Kiesler et al., 2015; MacDonald et al., 2011). Antigen-presenting cells and intestinal epithelium, in response to contact with pathogens at their basolateral surface, signal inflammation by expressing chemokines and cytokines, which attract and activate immune cells and directly activate lymphocytes (Goll and van Beelen Granlund, 2015; Okumura and Takeda, 2017). Epithelial cells and endothelium of venules in intestine express cell adhesion molecules, like CC chemokine ligand (CCL) 25 and mucosal addressin cell adhesion molecule-1 (MAdCAM-1), to attract T cells in response to inflammatory stimuli. The newly extravasated lymphocytes are activated and participate in immune reactions, produce more proinflammatory cytokines and regulate intestinal permeability, which in part

can lead to increased translocation of pathogens to mucosa and increased immune cell recruitment and activation. Inappropriate regulation in these functions can lead to an exaggerated and uncontrolled proinflammatory cycle, like seen in inflammatory bowel diseases (IBD). (MacDonald et al., 2011)

2.1.3 Inflammatory bowel diseases

Inflammatory bowel diseases are chronic and multifactorial diseases, which manifest in recurring and often debilitating inflammation of the gastrointestinal tract (Ramos and Papadakis, 2019). The two main forms of IBD are Crohn's disease (CD) and ulcerative colitis (UC). In UC, the inflammation and the associated ulcers are limited to the lining of the large intestine. In CD, the inflammation can affect any part of the gastrointestinal tract but is most common in ileum, and the inflammation and damage may reach deeper into the tissue forming difficult to treat fistulas. The incidence of IBD is increasing, especially in the developed countries (Ramos and Papadakis, 2019).

Both genetic and environmental factors contribute to pathogenesis of IBD. Genetic susceptibility can be linked to various aspects of mucosal protection and immune activation and regulation (Ramos and Papadakis, 2019). Microbial dysbiosis, which is promoted by Western lifestyle can enrich bacterial species, which weaken the mucosal barrier. Disruption of the epithelial barrier, provoked by either genetic factors or altered microbiome, is a critical step for the development of aberrant inflammation, and can lead to inappropriate immune reactions to commensal bacteria. Impaired immune responses, including macrophage activation and recruitment of neutrophils, allow bacterial translocation to mucosa in CD, whereas overemphasized T-cell response leads to overexaggerated inflammation in IBD. Naïve T helper (Th) cells can differentiate to various distinctive subsets, including Th1, Th2, Th17 and regulatory T cells (Tregs), according to their differentiation environment.

Unlike cytotoxic T cells which directly induce apoptosis of infected cells, Th cells secrete cytokines and augment the function of other immune cells. Phagocytotic immune cells promote Th1 cell differentiation in response to intracellular pathogens, whereas Th2 differentiation is initiated to respond to extracellular pathogens. Th17 cells function at mucosal surfaces and attract other immune cells during bacterial and fungal infections. Tregs are a subtype of anti-inflammatory Th cells which suppresses immune responses and participates in maintaining homeostasis. CD and UC have typical distortions in T-cell populations. T cells of *lamina propria* of IBD patients are resistant to apoptosis (Gregoire et al., 2017). Regulatory T cells are under-represented in IBD, and inflammation is mediated by Th1 and Th17 type cells in CD and Th2 cells in UC, resulting in distinct cytokine profiles in the intestine. (Gregoire et al., 2017; Kmiec et al., 2017; Ramos and Papadakis, 2019)

Pharmacological treatments for IBD are aimed to achieve and maintain remission in patients, improve quality of life, and avoid malignancies and surgical treatments (Sales-Campos et al., 2015). Conventional treatments include anti-inflammatory aminosalicylates and corticosteroids, immunosuppressive thiopurines and methotrexate, and antibiotics. The most effective of these treatment options, thiopurines and corticosteroids, may cause difficult side effects limiting their use. Biologic drugs include antibodies against proinflammatory cytokines, TNFα and IL-12, and leukocyte adhesion molecules. Although effective, they are also expensive, and development of drug resistance is common. Novel therapies that have gained approval for treatment of IBD include cell therapy using mesenchymal stromal cells, and Janus kinase (JAK) inhibitors.

2.1.4 Experimental colitis models

Several experimental models of colitis have been developed for mice and rats for the study of human IBD. Although none of them can quite recapitulate the

complexity of human disease, they are valuable tools in the study of mechanisms of IBD and colitis (Chassaing et al., 2014). Among the most common models are the chemically induced colitis models, including dextran sodium sulfate (DSS)- and trinitrobenzene sulfonic acid-induced colitis. Immune-relevant models include genetic IL-10 knockout model and infectious models. Examples of the commonly used colitis models are listed in Table 1. (Kiesler et al., 2015)

Table 1. Examples of chemically induced, genetic, infectious and other colitis models. SAMP1/Yit(Fc) is a mouse strain which develops a spontaneous ileitis. DSS = Dextran sodium sulfate, IL-10 = Interleukin 10, Muc2 = Mucin 2, TNBS = Trinitrobenzene sulfonic acid.

Chemical models	**Mechanism**	**Reference**
DSS	Epithelial barrier defect	Okayasu et al., 1990
TNBS	Excessive T-cell response	Neurath et al., 1995
Piroxicam	Oxidative stress	Berg et al., 2002
Oxazolone	Excessive T-cell response	Boirivant et al., 1998
Acetic Acid	Epithelial barrier defect	MacPherson and Pfeiffer, 1978
Genetic models	**Mechanism**	**Reference**
Muc2	Epithelial barrier defect, spontaneous colitis	Velcich et al., 2002, Van der Sluis et al., 2006
SAMP1/Yit(Fc)	Spontaneous colitis	Kosiewicz et al., 2001
Infectious models	**Mechanism**	**Reference**
IL-10 knockout	Regulatory and effector T cell imbalance	Berg et al., 1996
Salmonella spp.	Infectious colitis	Barthel et al., 2003
Other models	**Mechanism**	**Reference**
Adherent–invasive Escherichia coli	Infectious colitis	Boudeau et al., 1999
Adoptive transfer	Regulatory and effector T cell imbalance	Powrie et al., 1993

2.1.4.1 Dextran sodium sulfate model

DSS-induced colitis is one of the most used experimental colitis models of UC due to its simplicity and reproducibility (Eichele and Kharbanda, 2017), and is the model of choice in this thesis. DSS used in induction of colitis is a polymer chain consisting of (1 -> 6)-linked dextran chain with two to three sulphate moieties at the 2nd, 3rd or 4th carbons (Figure 2). Polymers weighing approximately 40 kDa are most colitogenic (Kitajima et al., 2000). DSS is a

stable polymer (Kitajima et al., 2002) which can be administered to animals in drinking fluid as 1-5% solution for a few days or longer to induce acute colitis (Okayasu et al., 1990). Cycles of low-concentration DSS and pure water scheme can be used to induce chronic colitis and eventually colorectal cancer (Perše and Cerar, 2012).

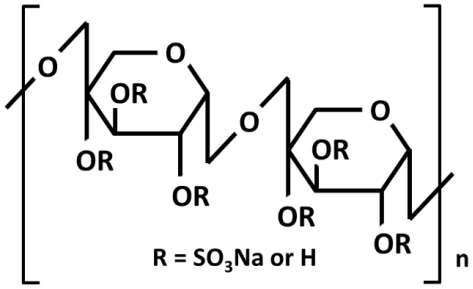

Figure 2. Chemical formula of dextran sodium sulfate used in colitis induction.

DSS is toxic to intestinal epithelial cells (Eichele and Kharbanda, 2017), and it increases intestinal permeability by loss of tight junction proteins and disrupts the epithelial barrier by increasing apoptosis rate in colonic epithelium (Mennigen et al., 2009; Poritz et al., 2007). The epithelial damage exposes mucosa to luminal bacteria and antigens and causes inflammation that is largely mediated by innate immunity but involves adaptive immunity as well (Kiesler et al., 2015; Perše and Cerar, 2012). Therefore, DSS model is especially useful for studying macrophage and neutrophil functions, mechanisms of innate immunity and epithelial barrier function in the involvement of intestinal inflammation. Bacterial translocation is essential to DSS-induced colitis, and in addition, it causes dysbiosis to some extent (Kiesler et al., 2015; Perše and Cerar, 2012). The damage in acute colitis can be lessened with antibiotics (Hans et al., 2000) and it is much milder in germ-free animals (Hudcovic et al., 2001). DSS induces the production of several

proinflammatory cytokines, e.g. tumor necrosis factor-α (TNF-α), IL-1β, interferon-γ (IFN-γ), IL-10, and IL-12 (Yan et al., 2009), of which IL-1β is the primary proinflammatory cytokine (Kiesler et al., 2015). DSS is taken up by macrophages in the mucosa and delivered to mesentery lymph node and eventually excreted to feces and urine (Perše and Cerar, 2012).

DSS induces diarrhea, weight loss, damage in the colon mucosa and bleeding. The magnitude of the damage depends on the dose and duration of administration. In acute inflammation, histopathological changes include crypt and surface epithelial damage, disappearance of goblet cells or crypts, ulceration and erosion of mucosa and inflammatory infiltrate of neutrophils in mucosa and submucosa. The typical changes in acute colitis are presented in Table 2. Chronic inflammation develops over a few weeks after the initial DSS administration and can be transmural. (Perše and Cerar, 2012) The susceptibility and involvement of T cells in DSS colitis is strain dependent. C57BL/6 mice develop more severe colitis than BALB/c and develop chronic colitis after DSS withdrawal, whereas BALB/c mice eventually recover (Melgar et al., 2005), making different strains more suitable in studying the healing process or chronic colitis. Strains also differ in their adaptive immunity response to DSS. Acute DSS colitis in C57BL/6 is polarized to Th1 and in BALB/c to Th2/Th17 response (Yang et al., 2017).

Table 2. Typical phenotypical, histopathological and biochemical changes in acute DSS colitis. IFNγ = Interferon-γ, IL = Interleukin, TNFα = Tumor necrosis factor-α.

Macroscopic changes	**Histopathological changes**	**Biochemical alterations**
Weight ↓	Loss of crypts and goblet cells	IL-1β, TNFα, IFNγ, IL-10, IL-12 ↑
Diarrhea	Apoptosis of epithelial cells	Tight junction proteins ↓
Intestinal permeability ↑	Erosions and ulcerations	Myeloperoxidase activity ↑
	Inflammatory cell infiltrate	

2.1.4.2 Other colitis models

Trinitrobenzene sulfonic acid (TNBS) colitis model is another commonly used chemically induced colitis model. TNBS is a hapten, which renders proteins immunogenic to the host when administered intrarectally, and induces diarrhea, weight loss and infiltration of neutrophils and macrophages (Neurath et al., 1995). TNBS colitis has a major Th1-mediated adaptive immunity component, but innate immunity reactions are also necessary for its induction. Compared to DSS colitis, TNBS induces more prominent Th cell infiltration, and the inflammation is generally transmural, leading to fibrosis in its chronic form. The similarity of cytokine responses and fibrotic activity between TNBS colitis and CD is why it is often used as model for CD. Oxazolone is another hapten given intrarectally (Boirivant et al., 1998), which induces natural killer cell-mediated mucosal inflammation by cytotoxic activity to intestinal epithelial cells and increase of intestinal permeability by production of IL-13 and can be used as a model of UC. (Antoniou et al., 2016; Kiesler et al., 2015)

In adoptive transfer colitis (Powrie et al., 1993), naïve Th cells are transferred to immunodeficient mice without capacity to produce mature T or B cells. Without regulatory T cells to counterbalance the actions of Th cells, mice develop colitis, which makes this model suitable to study immunoregulation, especially regulatory T cells. IL-10 knockout mice develop spontaneous colitis due to disturbed immunoregulation, which can further be aggravated by piroxicam (Berg et al., 1996; Berg et al., 2002). Piroxicam and indomethacin are non-steroidal anti-inflammatory drugs, which can also be used on their own to induce colitis. (Kiesler et al., 2015; Valatas et al., 2015)

Rectal administration of acetic acid causes epithelial injury and ulceration (MacPherson and Pfeiffer, 1978) and can be useful in studying mechanisms involving reactive oxygen species. Several other less widely used chemically induced (e.g. dinitrobenzene sulfonic acid), genetic (*mucin 2* knockout) and infectious (*Salmonella* spp.) models exist to study intestinal inflammation.

Most models require an intact microbiome to develop, as germ-free mice are resistant to many colitogenic factors, highlighting the significance of disturbed host-microbial interactions and epithelial barrier function in intestinal inflammation. (Kiesler et al., 2015; Low et al., 2013)

Intestine acts as a physical and an immunological barrier against microbes and dietary antigens, protecting the host from pathogens, while mediating tolerance to commensal bacteria and harmless antigens. This process is called immune homeostasis and its disruption can lead to inappropriate and chronic immune activation with devastating consequences to the host.

2.2 Renin-Angiotensin system

The renin-angiotensin system (RAS) is a complex network which regulates various physiological and pathophysiological functions in several organs (Fandriks, 2011; Paul et al., 2006). Systemic RAS is a critical regulator of blood pressure and it is involved in inflammation, apoptosis and fibrosis (Paul et al., 2006) (Figure 3). Angiotensinogen is the precursor of all peptide hormones in RAS, which is mainly produced in the liver. Kidneys produce and secrete a serine protease renin which cleaves ten N-terminal amino acid residues from angiotensinogen to produce angiotensin I (Ang I) (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu). RAS regulates blood-pressure via the classical RAS pathway, in which angiotensin-converting enzyme (ACE), expressed richly in pulmonary vascular endothelium, cleaves two C-terminal residues from angiotensin I to form angiotensin II (Ang II). Ang II acts through G-protein coupled receptors angiotensin II receptor 1 (AT1R) and to a lesser extent angiotensin II receptor 2 (AT2R). AT1R signaling facilitates an increase in blood pressure by direct and indirect fluid retention in the kidney by sodium excretion and stimulation of aldosterone secretion, and induces vasoconstriction in the vascular smooth muscle cells, especially in the splanchnic circulation (Fandriks, 2011). AT1R signaling leads to activation of nuclear factor-kappa B (NF-κB), the master regulator of inflammatory processes, leading to cytokine production, in several organs, leukocyte recruitment, apoptosis, cell proliferation and fibrosis. AT2R opposes the effects of AT1R and mediates vasodilatation, fluid excretion in kidney and intestine, and antiproliferation. In the alternative RAS pathway, Ang II is cleaved into angiotensin 1-7 (Ang 1-7) by angiotensin-converting enzyme 2 (ACE2), which is partially homologous with ACE but has distinct targets. Ang 1-7 acts through Mas receptor which facilitates similar effects as AT2R, thus also opposing the effects of AT1R signaling. ACE2 can also counter the effects of classical RAS by cleaving angiotensin I into angiotensin 1-9 which is further processed into Ang 1-7 by ACE and neprilysin (Fandriks, 2011). In addition to

these factors, many other enzymes produce and inactivate various less-known angiotensin peptides that act via their own receptors. (Fleming, 2006; Fyhrquist and Saijonmaa, 2008; Lavoie and Sigmund, 2003; Paul et al., 2006)

ACE has diverse known, and undoubtedly unknown, substrates relevant to function of RAS locally in various tissues. RAS is linked to kinin-kallikrein system in so that kallikrein can activate prorenin and ACE can inactivate the vasodilating peptide bradykinin. An alternative way of producing Ang II is by chymase, which can cleave Ang I into Ang II in various tissues. In addition to its enzyme activity, ACE acts as a surface receptor, which is activated by at least bradykinin as its natural substrate. Activation of ACE receptor involves dimerization of ACE and intracellular phosphorylation by casein kinase 2, which promotes retention of ACE at the cellular membrane (Kohlstedt et al., 2006; Kohlstedt et al., 2002), Activation of ACE receptor function leads to activation of c-Jun N-terminal kinase and subsequently c-Jun transcription factor, which upregulates various target genes, including cyclo-oxygenase-2 and ACE itself (Kohlstedt et al., 2004; Kohlstedt et al., 2005). (Bernstein et al., 2013; Fleming, 2006)

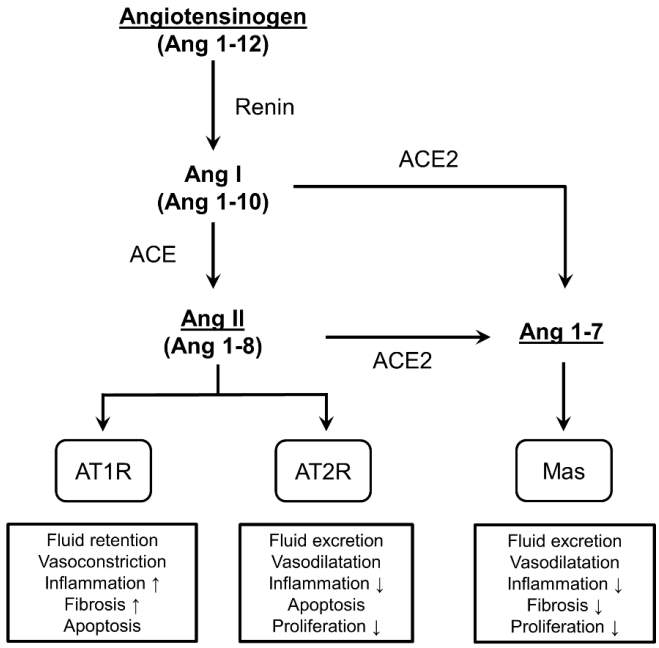

Figure 3. A simplified scheme of the main classical and alternative renin-angiotensin system pathways and effects. (Modified from Holappa et al., 2017)

2.2.1 Soluble and membrane-bound angiotensin-converting enzyme

ACE is a membrane-bound protein, which exists in somatic form in endothelial, epithelial and immune cells, and in germinal form only in spermatozoa (Ehlers et al., 2012). In addition, ACE can be enzymatically solubilized or shedded from the cell surface and can be found in plasma and other body fluids, but the membrane-bound form is considered more important for blood pressure regulation. In fact, the purpose of ACE shedding is not known, but has been suggested to reduce local production of Ang II by the release of the enzyme. However, soluble ACE is still enzymatically active and might serve some yet unknown function. The plasma ACE is derived mainly from the lung vascular endothelium, where it can be considerably

reduced during sepsis and acute lung injury (Orfanos et al., 2000; Votta-Velis et al., 2007). Inhibition of ACE, certain inflammatory diseases, like sarcoidosis, and lung injury lead to upregulation of soluble ACE in plasma, in which case the origin might be monocytic cells (Ehlers et al., 2012; Forslund et al., 1982). (Bernstein et al., 2013; Ehlers et al., 2012)

The somatic ACE consists of two, N- and C-terminal, extracellular domains with catalytic sites, a stalk region containing a proteolytic cleavage site near a transmembrane region, and a cytoplasmic domain with phosphorylation sites (Ehlers et al., 2012). A disintegrin and a metalloprotease 9 (ADAM9) was shown to cleave ACE from human umbilical vein endothelial cell membranes in response to proinflammatory stimuli, TNFα signaling and NF-κB activation (English et al., 2012). Both ACE and ADAM9 are expressed in endothelial cells, monocytes and macrophages, and therefore shedding might occur in various cell types (English et al., 2012). Active metalloprotease-dependent shedding of ACE involves conformational changes due to dephosphorylation of the intracellular tail (Kohlstedt et al., 2002), protein kinase C signaling, and is possibly dependent on resolution of dimerization of ACE, whereas regulation and mechanisms of basal shedding are yet unknown (Balyasnikova et al., 2002; English et al., 2012).

2.2.2 Inhibition of renin-angiotensin system

Due to the crucial role of RAS in the development of hypertension and associated cardiovascular diseases, inhibition of Ang II signaling is pivotal in the treatment of these diseases. Two major classes of pharmacologic RAS inhibitors are widely used to treat hypertension; ACE inhibitors and angiotensin II receptor blockers (ARBs). ACE inhibitors prevent the cleavage of Ang I into Ang II, and also bradykinin breakdown. In addition to bradykinin, some ACE inhibitors can trigger ACE receptor signaling (Fleming, 2006). ARBs are antagonists of AT1R and prevent receptor activation and

signal transduction without affecting Ang II levels. Third class of RAS inhibitors are renin inhibitors, which inhibit the formation of Ang I from angiotensinogen. ACE inhibitors and ARBs are generally well-tolerated drugs, but common adverse effects include hypotension and hyperkalemia, and cough in the case of ACE inhibitors. Rare and serious adverse effects include acute renal failure and liver damage, and in some cases, they may cause tissue swelling called angioedema, which usually affects tongue or pharynx but is also reported to rarely affect the intestine (Malde et al., 2007; Wilin et al., 2018).

In addition to pharmacologic compounds, nutrition-derived compounds have found to inhibit ACE activity (Jäkälä and Vapaatalo, 2010). Microbial fermentation of milk casein by lactic acid bacteria or by enzymatic processing produces bioactive peptides with antihypertensive properties, which have been verified in clinical trials (Cicero et al., 2013; Turpeinen et al., 2013; Xu et al., 2008), but the antihypertensive mechanism of action is not accounted to ACE inhibition alone (Majumder and Wu, 2014), and involves vasodilatation by increased Ang 1-7 and Mas receptor activation (Ehlers et al., 2011). Ile-Pro-Pro and Val-Pro-Pro are among these peptides, and they competitively inhibit, albeit weakly, ACE with 5 and 9 μM IC_{50} values, respectively (Lehtinen et al., 2010; Nakamura et al., 1995). Pharmacological ACE inhibitors captopril, and the active metabolite of enalapril, enalaprilat, inhibit ACE with respective IC_{50} values of 21 nM (Hooper and Turner, 1987) and 1.94 nM (Ceconi et al., 2007).

2.2.3 Renin-angiotensin system in the intestine

All the core enzymes and receptors of RAS (renin, angiotensinogen, ACE, ACE2, AT1R, AT2R, Mas receptor) are expressed in the gastrointestinal tract (Bernardi et al., 2012; Hamming et al., 2004; Hirasawa et al., 2002; Sechi et al., 1993) (Figure 4), where their functions include involvement in motility, transport of fluid, electrolytes, and nutrients, and participation in regulation

of inflammation (Fandriks, 2011; Fishlock and Gunn, 1970). Renin is produced by goblet cells and excreted into intestinal lumen with mucus in small intestine (Shorning et al., 2012). In colon, renin is localized into surface epithelium, smooth muscle cells and mesenchymal cells (Hirasawa et al., 2002). Angiotensinogen is produced locally in vascular smooth muscle cells, while Ang I and Ang II localize in epithelial cells especially in the crypts (Hume et al., 2016; Shorning et al., 2012). ACE is highly expressed in the brush border of small intestine, and in colon in lower quantities in epithelium, endothelial cells, and mesenchymal cells (Hirasawa et al., 2002; Shorning et al., 2012). Cleavage of ACE has been documented from human small intestine (Naim, 1996), but at the brush border the metalloprotease activity leading to shedding of ACE has been reported to be low (Oppong and Hooper, 1993). Soluble N-terminal domain of ACE has been found in intestinal lumen and is potentially processed from ACE post-shedding (Deddish et al., 1994). ACE2 is expressed in vascular endothelium, smooth muscle cells and the small intestine brush border (Hamming et al., 2004). AT1R is highly expressed in enterocytes, as well as vascular endothelium and mesenchymal cells in *lamina propria* (Hirasawa et al., 2002; Shorning et al., 2012). AT2R is expressed at much lower quantities, predominantly in the smooth muscle cells and vascular endothelium and to an even lesser extent on the luminal membranes of endothelial cells, crypts and mesenchymal cells (Hirasawa et al., 2002; Sechi et al., 1993). Expression of Mas receptor is low in healthy colon (Khajah et al., 2016).

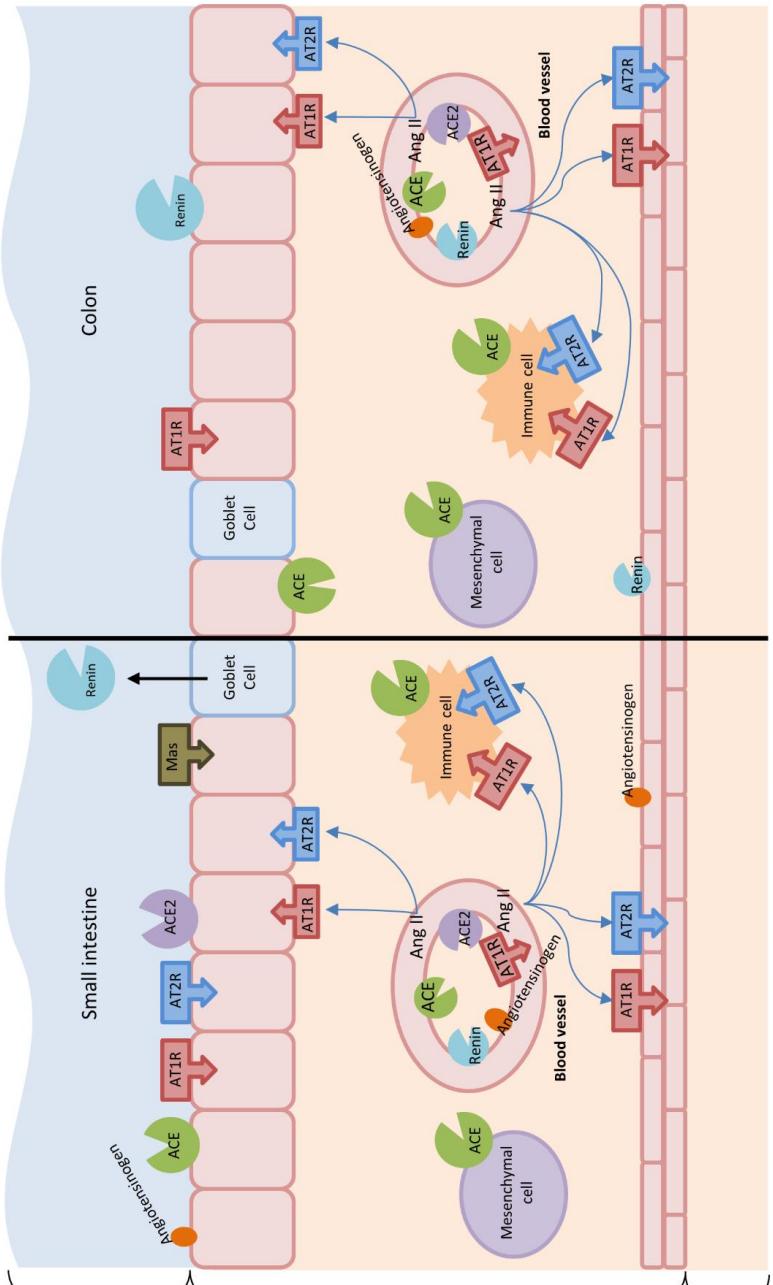

Figure 4. Illustration of the main components of renin-angiotensin system in small intestine and colon as currently known (modified from Paul et al., 2006 and Garg et al., 2012). ACE = Angiotensin-converting enzyme, ACE2 = Angiotensin-converting enzyme 2, Ang II = Angiotensin II, ATr = Angiotensin II receptor 1, AT2R = Angiotensin II receptor 2.

2.2.4 Renin-angiotensin system in the pathophysiology of intestinal inflammation

RAS is involved in various pathophysiological functions in the progress of intestinal inflammation and Ang II is the key proinflammatory factor in these processes via AT1R. Ang II production is increased in intestinal inflammation (Jaszewski et al., 1990; Khajah et al., 2016), and in some cases its local concentration can act as a switch to modulate signaling between physiological and pathophysiological conditions (Hornych et al., 1973; Jin et al., 1998). Pronounced AT1R signaling leading to NF-κB activation regulates similar inflammatory responses in intestine as it does systemically (Garg et al., 2012; Lee et al., 2014; Wang et al., 2013). Ang II induces apoptosis and proliferation of intestinal epithelial cells, induces adhesion molecule expression in mucosal venules leading to leukocyte recruitment, and fibrosis in a healing intestinal wall (Garg et al., 2012; Wang et al., 2013). Ang II induces contractions in the smooth muscles of the intestine (Fishlock and Gunn, 1970; Garg et al., 2012). High concentrations of Ang II inhibit sodium and fluid absorption via AT1R, although low concentrations stimulate it via AT2R (Hornych et al., 1973; Jin et al., 1998), which likely contributes to diarrhea in intestinal inflammation. Additionally, Ang II enhances and activates proinflammatory leukocyte functions like cytokine production and cytotoxicity in macrophages and neutrophils *in vivo* (Aki et al., 2010; Khan et al., 2017) and dendritic cells, and T cells *in vitro*, and they all prominently express AT1R on their surface (Khan et al., 2017; Meng et al., 2017; Zhang and Crowley, 2013). At least macrophages and dendritic cells also express ACE and produce Ang II (Chen et al., 2013b; Danilov et al., 2003).

2.2.5 Inhibition of renin-angiotensin system in experimental colitis

Due to the extensive role RAS plays in regulation of inflammation, pharmacological modulation of Ang II signaling has received considerable interest as a potential treatment for intestinal inflammation. In animal experiments, ACE inhibitors, captopril, enalapril, enalaprilat and lisinopril, (Jahovic et al., 2005; Koga et al., 2008; Lee et al., 2014; Okawada et al., 2016; Spencer et al., 2007; Sueyoshi et al., 2013; Wengrower et al., 2004) and angiotensin II receptor blockers, losartan and its analogues, valsartan, candesartan, olmesartan and telmisartan (Arab et al., 2014; Inokuchi et al., 2005; Liu et al., 2016; Mizushima et al., 2010; Nagib et al., 2013; Okawada et al., 2011; Okawada et al., 2016; Santiago et al., 2008; Shi et al., 2016; Wengrower et al., 2012) have all alleviated experimental colitis and histological damage in DSS, TNBS and IL-10 knockout colitis models (**Error! Reference source not found.**3). Both ACE inhibition and angiotensin II receptor blockade reduce epithelial cell apoptosis and fibrosis in colon (Jahovic et al., 2005; Liu et al., 2016; Okawada et al., 2011; Spencer et al., 2007; Wengrower et al., 2012; Wengrower et al., 2004). On the contrary, inhibition of Mas receptor or administration of Ang II exacerbates colitis, but administration of low dose Ang 1-7 prevents and ameliorates DSS colitis (Khajah et al., 2016; Shi et al., 2016).

The role of endogenous RAS in colitis has also been studied in genetically modified animals. Angiotensinogen knockout mice develop milder colitis and they have impaired IL-1β and IFN-γ and higher IL-10 production, similar to losartan-treated mice (Inokuchi et al., 2005). Also AT1R knockout protects mice from DSS colitis and the mice have lower TNFα production than their wildtype counterparts (Katada et al., 2008; Mizushima et al., 2010). Conversely, renin overexpression dramatically worsens TNBS colitis (Shi et al., 2016).

In addition to colitis, local RAS has been studied in context of other diseases of the gastrointestinal tract. In a rat model of short-bowel syndrome, ACE inhibitors attenuated epithelial cell apoptosis in the small intestine (Wang et al., 2013). ACE inhibitor lisinopril, the ARB losartan and Ang 1-7 have accelerated the healing of gastric ulcers in rats (Pawlik et al., 2016).

Table 3. Preclinical studies of ACE inhibitors, angiotensin II receptor blockers, and renin inhibitors and genetic models in colitis conducted using mice or rats. Mouse strains are C57/BL6, AT1R -/- C57BL/6, IL10 -/- C57BL/6, ICR, Agt -/- ICR, and BALB/c. Rat strains are Sabra, Sprague-Dawley, and Wistar rats. DAI = disease activity index, DCL = deschlorolosartan, DSS = dextran sodium sulfate, L = Losartan, RenTgMK = Mice constitutively overexpressing renin, TNBS = Trinitrobenzene sulfonic acid.

ACE inhibitors

Treatment	Dose	Colitis model	Strain	Outcome	Reference
Captopril	50 mg/kg/d	TNBS	Sabra	Reduction of DAI, histopathology and fibrosis	Wengrower et al., 2004
Captopril or lisinopril	0.1 or 1 mg/kg/d	TNBS	Sprague-Dawley	Reduction of macroscopic and histopathology scores, and collagen deposition by captopril	Jahovic et al., 2005
Enalaprilat	14.5 µg/d	DSS	C57BL/6	Improved weight, histopathology, reduced apoptosis rate	Spencer et al., 2007
Enalaprilat	14.5 or 145 µg/d	DSS	C57BL/6	Increased survival, reduced DAI, histopathology and fibrosis	Koga et al., 2008
Enalaprilat	12.5 mg/kg/d	Piroxicam	IL10 -/- C57BL/6	Increased survival, reduced disease score. Improved histopathology	Sueyoshi et al., 2013
Enalapril	1 or 5 mg/kg/d	DSS	C57BL/6	Improvement in histopathology, reduced NF-κB activation and proinflammatory cytokines	Lee et al., 2014
Enalaprilat	25 mg/kg/d	DSS	IL10 -/- C57BL/6	Improved weight and histopathology, reduced NF-κB activation and proinflammatory cytokines	Okawada et al., 2016

Angiotensin II receptor blockers

Treatment	Dose	Colitis model	Strain	Outcome	Reference
Valsartan	160 mg/ml	DSS & TNBS	Sprague-Dawley	Improved weight, and histopathology and macroscopic score in TNBS model, reduced diarrhea in DSS model	Santiago et al., 2008
Candesartan	0.4 mg/kg/d	DSS	C57BL/6	Improved weight, histopathology and TNFα in AT1R-/-, improved disease activity and body weight by candesartan	Mizushima et al., 2010
Losartan, candesartan, deschloro-losartan	L/DCL 100 mg/kg/d candesartan 10 mg/kg/d	Piroxicam	C57BL/6	Improved histopathology and reduced epithelial cell apoptosis by all treatments	Okawada et al., 2011
Losartan	7 mg/kg/d	TNBS	Sabra	Reduced TGF-β-mediated fibrosis by losartan	Wengrower et al., 2012

Olmesartan	1, 3 or 10 mg/kg/d	DSS	Wistar	Improved colon length, histopathology and TNFα	Nagib et al., 2013
Telmisartan	10 mg/kg/d	TNBS	Wistar	Improved weight, DAI and macroscopic and histopathology scores	Arab et al., 2014
CCG-203025 (losartan analog)	100 mg/kg	DSS & piroxicam	IL10 -/- C57BL/6	Improved weight and histopathology, reduced proinflammatory cytokines in DSS model, improved weight in piroxicam model	Okawada et al., 2016
Losartan	10 mg/kg/d	TNBS	C57BL/6	Improved weight and histopathology and macroscopic scores, reduced apoptosis	Liu et al., 2016
Losartan	30 μg/kg/d	TNBS	ICR	Milder colitis, impaired IL-1β and IFNγ production	Inokuchi et al., 2005
-	-	Ang II infusion + TNBS	C57BL/6	Ang II infusion caused more weight loss, higher histopathology and macroscopic scores, and proinflammatory cytokine production.	Shi et al., 2016
Losartan	11 mg/ml (~15 mg/kg/d)	TNBS	C57BL/7	Improved weight, histopathology and macroscopic scores	Shi et al., 2016

Genetic models

Animal model	Treatment	Colitis model	Strain	Outcome	Reference
Agt -/-	-	TNBS	Agt -/- ICR	Improved weight and histopathology, impaired IL-1β and IFNγ production	Inokuchi et al., 2005
AT1R -/-	-	DSS	AT1R -/- C57BL/6	Improved weight and histopathology, lower TNFα expression and production	Katada et al., 2008
AT1R -/-		DSS	AT1R -/- C57BL/6	Improved weight, histopathology and TNFα in AT1R-/-, improved disease activity and body weight by candesartan	Mizushima et al., 2010
Renin overexpression	Aliskiren 20 mg/kg/d	TNBS	RenTgMK 129SV	Reduced survival, worse histopathologic and macroscopic scores, increased apoptosis and proinflammatory cytokine production in RenTgMK mice. Aliskiren treatment improved all parameters.	Shi et al, 2016

Others

Treatment	Dose	Colitis model	Strain	Outcome	Reference
Aliskiren	3 or 10 mg/kg/d	DSS	C57BL/6	Improved weight, DAI, macroscopy, and histopathology at a dose of 10 mg/kg/d.	Patel et al., 2014
Angiotensin 1-7	0.01, 0.06, 0.1, 0.3 or 1 mg/kg/d	DSS	BALB/c	Doses of 0.01 and 0.06 mg/kg/day improved weight and histopathology and macroscopic scores	Khajah et al., 2016

2.2.6 Renin-angiotensin system in inflammatory bowel diseases

RAS is involved in several processes, which are important in the pathogenesis of IBD, including inflammation, apoptosis, fibrosis and recruitment of immune cells (Fandriks, 2011; Garg et al., 2012). However, compared to the accumulating evidence on the importance of RAS in experimental colitis, there are precious few studies of RAS in human IBD. Angiotensinogen expression was found to be low in ileum of CD patients (Hume et al., 2016). However, Ang I and Ang II levels were elevated in intestinal mucosa during active inflammation in CD (Jaszewski et al., 1990). Renin expression was increased in inflamed lesions of both UC and CD patients (He et al., 2019). Mas receptor expression was increased in colons of IBD patients, but inflammation lowered its expression, and CD increased ACE2 expression in colon (Garg et al., 2015c). Plasma ACE2 concentration and activity, renin and Ang 1-7 levels were higher in IBD patients than in healthy control subjects, while ACE and Ang II remained unchanged (Garg et al., 2015a; Garg et al., 2015c).

ACE inhibitors and ARBs are generally well-tolerated drugs and could offer a novel treatment option in IBD patients as well, but no controlled trials have been conducted on the efficacy of RAS inhibition in IBD, and none are in progress according to ClinicalTrials.gov. For now, there are only few clinical studies which have investigated ARBs or ACE inhibition in IBD patients. In one study, levels of proinflammatory cytokines in mucosal biopsies were compared between age and gender matched IBD patients who either were on ARB therapy or not (Shi et al., 2016). IBD patients on ARB medication had lower gene expression of several proinflammatory cytokines and chemokines, including *IL-1β*, *TNFα*, *IL-6*, *CCL2* and *CXCL8* (encoding IL-8), than those who were not taking ARBs (Shi et al., 2016). In one retrospective study, use of ACE inhibitors and ARBs in IBD patients was associated with lower risk of hospitalization, operations and corticosteroid medication (Jacobs et al., 2019).

In another similar study, use of ACE inhibitors or ARBs was associated with lower disease activity and fewer hospitalizations and need of operations in IBD patients (Garg et al., 2015b). Prospective clinical trials are needed to evaluate the potential of ACE inhibitors and ARBs in IBD.

Renin-angiotensin system is best known as the main regulator of blood pressure, but newly found local systems have revealed its role in many other physiological and pathophysiological functions. In the intestine, RAS is involved in many aspects of inflammation, which has prompted investigations on the possibility to use RAS inhibitors as treatment for intestinal inflammation.

2.3 Glucocorticoids

Glucocorticoids are steroid hormones that are crucial for anti-inflammatory immune responses, stimulate gluconeogenesis and fat breakdown for energy. Systemic glucocorticoid synthesis takes place in *zona fasciculata* of adrenal cortex in response to humoral control of hypothalamus and anterior pituitary. Hypothalamus produces corticotropin-releasing hormone and vasopressin, which together induce the pituitary to release adrenocorticotropic hormone (ACTH). ACTH activates melanocortin 2 receptor, which induces an increase of intracellular cyclic adenosine monophosphate (cAMP) to activate protein kinase A. This in turn activates the transcription factor, steroidogenic factor-1, to induce production of steroidogenic enzymes and stimulates the production of adrenal glucocorticoids. These inducible genes include cytochrome P450 enzymes (CYP) CYP17, CYP21B and CYP11B1, which is the final and rate-limiting enzyme in active glucocorticoid synthesis pathway (Li et al., 2004; Mueller et al., 2006) (Figure 5). Mouse adrenals do not express CYP17, and therefore some steps in the corticosteroid synthesis are replaced by 3β-hydroxysteroid dehydrogenase (HSD). Therefore, the active glucocorticoid in humans is cortisol and in mice and other rodents is corticosterone.

The anti-inflammatory mechanisms of glucocorticoids involve actions at every level of inflammation. Glucocorticoids act via the glucocorticoid receptor, a nuclear receptor which can, in addition to its function as a transcription factor, activate or inhibit other transcription factors and mediators of inflammation. They decrease signaling through cytokine and Toll-like receptors, T-cell receptor in T cells, and immunoglobulin E receptor in mast cells, dampening inflammatory responses and allergic reactions. Glucocorticoids reduce cytokine production by inhibiting the synthesis and activity proinflammatory transcription factors, like NF-κB, and by enhancing their breakdown. They reduce leukocyte recruitment by reducing chemokine production, adhesion molecule expression in vascular wall and inflammatory cells, and by decreasing vascular permeability. Finally, they can induce

apoptosis and affect the differentiation of lymphocytes, dendritic cells, macrophages and eosinophils. (Cain and Cidlowski, 2017)

Due to their broad anti-inflammatory effects, synthetic glucocorticoids are used in pharmacologic treatment of various inflammatory conditions, like rheumatoid arthritis, asthma and IBD, as well as to suppress immune system after organ transplantation. Common synthetic glucocorticoids in pharmacological use include hydrocortisone, fluticasone, prednisone, dexamethasone and budesonide. Owing to their broad effects, glucocorticoid therapy causes a wide range of side effects, including hyperglycemia, osteoporosis, increased risk of infections, and mood changes, which limits their long-term use. However, dependence on glucocorticoid therapy, meaning inability to reduce glucocorticoid dose, is common in patients, and a small part of patients is resistant to the benefits of glucocorticoid therapy altogether. (De Iudicibus et al., 2011; Vandewalle et al., 2018).

2.3.1 Glucocorticoids in the intestine

Intestine expresses its own fully functional steroid synthesis network and is able to produce anti-inflammatory glucocorticoids to regulate immune homeostasis (Bouguen et al., 2015b; Cima et al., 2004; Sidler et al., 2011). In the intestine, glucocorticoids are produced at the base of the crypts (Atanasov et al., 2008; Huang et al., 2014), under the control of the transcription factor liver receptor homolog-1 (LRH-1), instead of steroidogenic factor-1 in the adrenals (Bouguen et al., 2015b; Mueller et al., 2007; Mueller et al., 2006). LRH-1 induces the production of steroidogenic enzymes cholesterol side-chain cleavage enzyme, encoded by *CYP11A1* gene, 11β-hydroxylase, encoded by *CYP11B1*, 3β-HSD and 11β-HSD (Bouguen et al., 2015b; Cima et al., 2004) (Figure 5). Unlike in adrenals, cAMP inhibits glucocorticoid synthesis in intestine, where it seems to be activated by protein kinase C (Mueller et al., 2007). Intestinal glucocorticoid synthesis is stimulated by activation of cells of

innate and adaptive immune systems (Cima et al., 2004; Noti et al., 2010b). Acute inflammation stimulates T cells and macrophages to produce TNFα, which induces glucocorticoid synthesis in epithelial cells (Noti et al., 2010b). In chronic inflammation, however, TNFα activation of c-Jun and NF-κB inhibits glucocorticoid production by reducing *Lrh-1* and *Cyp11b1* expression in mice (Huang et al., 2014).

The known functions of locally produced glucocorticoids in the intestine are still sparse, but they all involve anti-inflammatory regulation of immune system. Intestinal glucocorticoids are an important feedback mechanism in regulation of intestinal inflammation (Bouguen et al., 2015a; Coste et al., 2007). They can directly inhibit antigen-dependent activation of T-cells and promote apoptosis in them (Cima et al., 2004; Sidler et al., 2011). Intestinal glucocorticoids also contribute maintaining immune homeostasis by promoting the expression the anti-inflammatory and tolerogenic transcription factor, peroxisome proliferator activated receptor gamma (PPARγ) (Annese et al., 2012; Bouguen et al., 2015b).

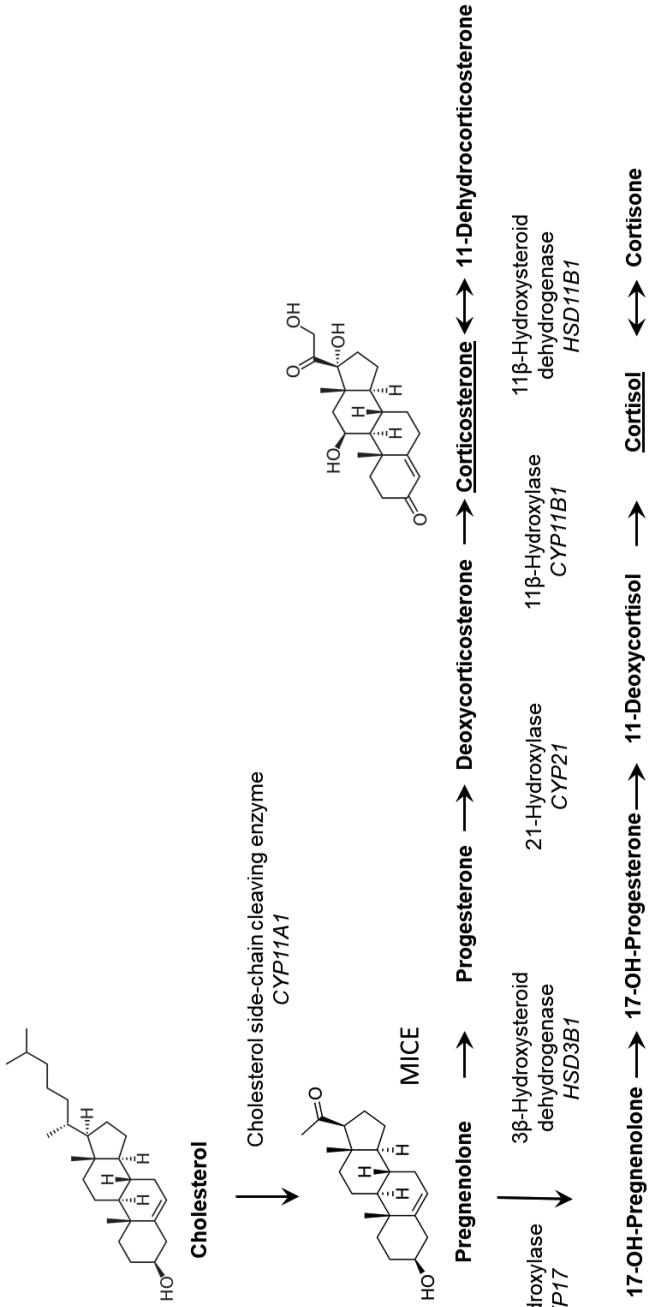

Figure 5. Glucocorticoid synthesis pathway in human and mice.

2.3.2 Intestinal glucocorticoids in inflammatory bowel diseases

As glucocorticoids are the key anti-inflammatory hormones, it is no surprise that perturbations in their local synthesis is involved in the pathogenesis of IBD in many ways. LRH-1, CYP11A1, CYP11B1, and HSD11B1 expression, and consequently the levels of glucocorticoids, are low in inflamed colon samples of IBD patients (Ahmed et al., 2019; Bouguen et al., 2015b; Coste et al., 2007). T cells in intestine are resistant to apoptosis in IBD patients, but the connection between low glucocorticoid production and aberrant T-cell activation has not been established. The deficiency in glucocorticoid production also leads to reduced expression of PPARγ (Bouguen et al., 2015b), which is implicated in UC pathophysiology and is the target of 5-aminosalicylic acid, an anti-inflammatory drug used to treat IBD (Rousseaux et al., 2005). A single-nucleotide LRH-1 polymorphism has been identified to be associated with UC (Jostins et al., 2012). One potential relationship of glucocorticoids and IBD pathophysiology involves endoplasmic reticulum (ER) stress, which is associated with IBD (Fritz et al., 2011). Inflammation and other stressors in secretory cells, like intestinal epithelial cells, result in overload of protein-processing demand in the ER for its protein-folding capacity, and accumulation of unfolded proteins leads to ER stress, (Bouguen et al., 2015a; Fritz et al., 2011; Kaser et al., 2008). Glucocorticoids induce responses to augment protein folding and degradation during ER stress, thus relieving it (Das et al., 2013), but involvement of local glucocorticoid production has not been proven.

2.3.3 Interactions of renin-angiotensin system and glucocorticoids

Glucocorticoids and renin-angiotensin system are linked in adrenals and vasculature in many ways. Glucocorticoids increase expression of ACE (Fishel et al., 1995; Mendelsohn et al., 1982) and AT1R (Sato et al., 1994; Shelat et al., 1999) in various tissue types, including endothelium, vascular smooth muscle cells and leukocytes. Conversely, Ang II can induce and potentiate ACTH-activated adrenal glucocorticoid synthesis (McKenna et al., 1978; Parker et al., 1983; Spinedi et al., 1989). Although ACTH is the primary stimulating molecule of glucocorticoid synthesis, ACTH is only two to three times more potent activator than of Ang II (Pham-Huu-Trung et al., 1986).

Glucocorticoids are anti-inflammatory steroid hormones which are produced centrally in adrenal cortex but also locally in intestine. Intestinal glucocorticoid synthesis is regulated differently from adrenals, and its roles include maintaining immune homeostasis by promoting immune tolerance and inhibiting immune activation. Disturbances in intestinal glucocorticoid synthesis are associated with inflamed lesions in inflammatory bowel diseases.

2.4 Mesenchymal stromal cells

Mesenchymal stromal cells (MSCs) are multipotent stem cells, which possess immune-regulatory and anti-inflammatory properties. They are found in most tissues, including bone marrow, adipose tissue and umbilical cord, with the ability to differentiate into chondrocytes, osteoblasts and adipocytes (Dave et al., 2015). Upon activation, they can inhibit proinflammatory immune cell functions, like immune cell proliferation, maturation and migration as well as antigen presentation, and promote leukocyte differentiation towards anti-inflammatory and regulatory functions (Gregoire et al., 2017). Promoting immune cell differentiation to regulatory T cells, dendritic cells and macrophages leads to reduction of proinflammatory cytokines and elevation of anti-inflammatory mediators, like IL-10 and indoleamine 2,3-dioxygenase (IDO), which further upregulate regulatory T cells and inhibit other T cells. Additionally, MSCs themselves secrete growth factors and immunosuppressive compounds, like PGE2, nitric oxide and TGFβ (Gregoire et al., 2017). MSCs migrate to sites of inflammation where they are activated by the various factors, including proinflammatory cytokines IL-1β, TNFα and IFNγ to reduce inflammation and promote tissue healing and survival. Without a proinflammatory stimulus, MSCs can assume a proinflammatory phenotype, which promotes immune cell recruitment and polarization to proinflammatory subtypes. Thus, MSCs support both the pro- and anti-inflammatory functions of the host as needed. (Bernardo and Fibbe, 2013; Dave et al., 2015; Gregoire et al., 2017)

2.4.1 Mesenchymal stromal cell therapy

In addition to the anti-inflammatory and immunosuppressive properties of MSCs, they possess an unusual trait for being low in immunogenicity, which has made them a target of interest for allogenic cell therapy, in which the recipient receives donated cells from a third party. In fact, they were first

thought to be completely immune privileged, meaning they would not cause an immune reaction even when donor and host are not human leukocyte antigen matched, which would therefore enhance the safety of MSC therapy (Ankrum et al., 2014). However, recent studies have demonstrated an alternative mechanism for immune-suppression by MSCs, which requires host immune-cell reaction and subsequent phagocytosis of apoptotic MSCs to induce IDO production and immune cell differentiation towards regulatory types (de Witte et al., 2018; Galleu et al., 2017). In autologous cell transfer, the recipient is treated with their own cells and there are no similar immune reactions between host and transferred cells.

Cell therapy using MSCs has been investigated as a treatment in regenerative medicine, and for various immunologic and inflammatory conditions like graft-versus-host disease and IBD (Ankrum and Karp, 2010). Either autologous and allogenic MSCs are typically harvested from bone marrow or adipose tissue and expanded *in vitro* before administration to the patient. Although MSCs possess the capacity to differentiate into different cell types, in cell therapy, the main therapeutic effect is thought to be facilitated by their ability for immune regulation.

2.4.2 Mesenchymal stromal cells as a potential treatment for inflammatory bowel diseases

Allogenic, from the same species, and xenogeneic, from a different species, MSCs have been studied in context of intestinal inflammation in preclinical studies. MSCs have been effective in treating DSS and TNBS colitis when using either untreated, i.e. not activated, murine MSCs (Chen et al., 2013a; He et al., 2012; Sala et al., 2015; Tanaka et al., 2008; Wang et al., 2016; Xie et al., 2017) or human, i.e. xenogeneic, MSCs, which have been activated by IFNγ, IL-1β or Nucleotide Binding Oligomerization Domain Containing 2 (NOD2) prior to administration (Duijvestein et al., 2011; Fan et al., 2012; Kim et al., 2013). Non-

activated human MSCs have not as been effective in treating murine colitis (Duijvestein et al., 2011; Fan et al., 2012), likely due to lack of cross-reactivity between cytokines and their receptors between species (Galleu et al., 2017).

MSCs have been studied in the treatment of refractory IBD in numerous clinical trials (Gregoire et al., 2017; Lightner et al., 2018; Qiu et al., 2017). Cell therapy can be administered either intravenously to treat luminal IBD or applied topically to treat fistulas in CD. Phase I and II clinical trials on treating luminal disease with bone-marrow derived MSCs have demonstrated a somewhat inconsistent benefit, but in general allogenic transfer of MSCs has been more efficient at reducing disease activity and inducing remission than autologous transfers (Duijvestein et al., 2010; Forbes et al., 2014; Hu et al., 2016; Liang et al., 2012; Qiu et al., 2017). Local administration of adipose-tissue or bone-marrow derived MSCs for treatment of fistulas have been efficient in achieving fistula closure in both autologous and allogenic transfers (de la Portilla et al., 2013; Garcia-Olmo et al., 2009; Lee et al., 2013; Lightner et al., 2018; Molendijk et al., 2015; Panes et al., 2016). Alofisel™ (Panes et al., 2016) by TiGenix and Takeda, is the first MSC product to gain marketing approval for treatment of fistulas in Crohn's disease. At the moment, five trials are ongoing according to ClinicalTrials.gov for the treatment of fistulas in Crohn's disease and one for the treatment of ulcerative colitis using MSC therapy, all of them employing local instead of systemic application of MSCs. The inconsistent but promising results gained in clinical trials underline the importance of investigating optimal culture and treatment protocols in MSC therapy.

2.4.3 Glucocorticoids and renin-angiotensin system in mesenchymal stromal cell therapy

RAS and glucocorticoids are general proinflammatory and anti-inflammatory mediators, respectively, and as such, immunosuppressive MSCs interact with them in the host and sense the inflammatory environment through

angiotensin and glucocorticoid receptors, in addition to classical cytokines. In a radiation-induced proctitis model, MSCs were shown to increase colonic glucocorticoid production and express steroidogenic enzymes and produce glucocorticoids themselves (Bessout et al., 2014). Like activation by proinflammatory cytokines, pretreatment of MSCs with Ang II improved their beneficial effect in a model of myocardial infarction (Liu et al., 2015). On the other hand, microvesicles from MSCs were shown to downregulate the classical proinflammatory RAS and upregulate the alternative anti-inflammatory RAS in a model of pulmonary hypertension, in which the beneficial effects were mediated by Mas receptor (Liu et al., 2018).

Mesenchymal stromal cells are immunoregulatory cells with potential for immunomodulatory cell therapy due to their immunosuppressive and wound healing capacity, and their low immunogenicity. They have shown promise in the treatment of inflammatory bowel diseases, but optimal culture conditions and augmentation of MSC activation by cytokines and growth factors are under investigation in animal models. As the knowledge of involvement of renin-angiotensin system and local glucocorticoids in the process of intestinal inflammation is emerging, their connections with mesenchymal stromal cells are being realized.

3 AIMS OF THE STUDY

Renin-angiotensin system is involved in several aspects of inflammation and wound healing in the intestine. This thesis characterizes intestinal RAS during colitis, explores novel interventions for intestinal inflammation in preclinical studies with a link to the intestinal RAS, and investigates the possible role of RAS in regulation of glucocorticoid synthesis in the gut. All studies employ the DSS-induced colitis model in mice. In the first study (Study I), ACE shedding and glucocorticoid synthesis were localized in the inflamed gut. Associations between RAS and glucocorticoid synthesis were investigated in Studies I and II. Study III focused on examining and comparing how different RAS inhibitors could improve colitis. Safety and efficacy of mesenchymal stromal cell (MSC) therapy in DSS-induced colitis, and its effect on intestinal RAS were examined in Study IV.

The specific aims of this study were to:

- Test novel therapeutic interventions, inhibition of RAS by ACE inhibitors and angiotensin II receptor blockers and MSC therapy, for intestinal inflammation. (Studies III and IV)
- Analyze how intestinal inflammation, pharmacologic and dietary inhibition of RAS, and MSC therapy affect intestinal RAS components. (Studies I, II, III and IV)
- Investigate possible connections between local RAS and glucocorticoid synthesis in healthy and inflamed intestine. (Studies I, II and III)

4 MATERIALS AND METHODS

4.1 Experimental animals

BALB/c male mice aged 7-9 weeks were used in all the studies. The animals were obtained from the commercial breeders, Scanbur (Sollentuna, Sweden), for Studies I and III, and Envigo (formerly Harlan Laboratories) (Horst, Netherlands) for Studies II and IV. The animal experiments were approved by the National Animal Experiment Board of Finland (permits ESAVI/6314/04.10.03/2012 and ESAVI/114/04.10.07/2015). The mice were housed in pairs in a specific-pathogen free animal facility under a 12h/12h light/dark cycle at 22 ± 2 °C and 55 ± 15% humidity. They were given food (Teklad Global 18% Protein Rodent Diet, Harlan Laboratories) and drinks *ab libitum*. In Study III, the mice were housed in individually-ventilated cages.

4.2 Colitis models and study designs

In all the studies, acute colitis was induced to the colitis groups by adding 3% or 5% (w/v) 40kDa DSS (TdB Concultancy, Uppsala, Sweden) to the drinking fluid of the mice for 6 to 7 days. Mice receiving tap water for the same duration were used as healthy controls. During the experiments, mice were weighed to assess the severity of colitis, and their condition were observed daily.

The study designs are presented in Figure 6. In Study I, the effects of two different DSS concentrations were studied. The mice were divided into three groups and given either tap water or 3% or 5% DSS solution for 7 days, after which they were sacrificed for sample collection.

The intervention studies (II, III and IV) entailed groups of healthy and DSS-control mice with untreated colitis, and intervention groups receiving DSS and the test compounds to test the interventions' ability to alleviate colitis and

modulate intestinal RAS and glucocorticoid synthesis during intestinal inflammation. ACE inhibitors and ACE-inhibiting Ile-Pro-Pro were examined in Studies II and III. The test compounds (Table 4) were given in drinking water for 5 days before the introduction of DSS to acquaint the mice to the compounds, after which acute colitis was induced by adding 3% DSS to the drinking fluids containing the test compounds for another 7 days, and then sacrificed. In Study II, the mice received either tap water, 15.7 mg/l ACE inhibitor, captopril (PHR1307-1G, Sigma-Aldrich, St. Louis, MO, USA), or 833 mg/l ACE-inhibiting peptide, Ile-Pro-Pro (H-4632, Bachem, Bubendorf, Switzerland), as test compounds.

In Study III, the mice initially received either 11 mg/l ACE inhibitor, enalapril (PHR1289, Sigma Aldrich), 37 mg/l angiotensin II receptor blocker, losartan (PHR1602, Sigma Aldrich), or both for the first 5 days, after which the concentrations of enalapril and losartan were adjusted to 25 mg/l and 83 mg/l, respectively for the duration of the 7-day DSS challenge. The daily doses are presented in Table 4. The daily doses of medications were based on effective doses reported in publications of colitis studies (Jahovic et al., 2005; Lee et al., 2014; Liu et al., 2016), except for Ile-Pro-Pro, which had not been investigated in colitis studies before.

Table 4. Conversions of concentration of compounds in drinking fluid to estimation of daily doses per animal and weight during DSS administration. The numbers in brackets indicate the initial doses in Study III.

Study	Treatment	Dose		
		mg/l	mg/day	mg/kg/day
Study II	Captopril	15.7	0.11	5
	Ile-Pro-Pro	0.833	5	227
Study III	Enalapril	25 (11)	0.075 (0.033)	3(1.3)
	Losartan	83 (37)	0.25 (0.11)	10(4.4)

In Study IV, two separate animal experiments were conducted to test MSCs in acute colitis (Study IVa) and during tissue regeneration (Study IVb). Acute colitis was first induced to four groups of mice by administering 3% DSS for 7 days (Study IVa). The colitis groups received either vehicle (0.9 % NaCl + 5% human serum albumin) or 0.5 x 10^6 freshly cultivated, meaning they were not frozen between their expansion and administration, human bone-marrow derived, platelet-lysate expanded MSCs in vehicle intraperitoneally or intravenously via the tail vein under isoflurane anesthesia on the 3rd and 5th days of DSS administration. To dilate the tail veins, the mice were kept in a warming cabinet at 37 °C for 5-10 minutes prior to inducing general anesthesia with isoflurane gas. The tails of the mice receiving intravenous injections were warmed on a heat pad while MSCs or vehicle were injected to the tail vein using a G30 needle. The animals were sacrificed after receiving DSS for 7 days.

Next, colitis was induced for three additional groups with 3% DSS for 6 days, after which they received tap water for an additional 7 days to allow healing process to begin (Study IVb). The colitis groups received either vehicle (0.9% NaCl + 3.6% human serum albumin) or 0.5 x 10^6 freshly cultivated or cryopreserved, meaning frozen after expansion and thawed just before administration, MSCs intravenously. The MSCs were cultivated at Advanced

Cell Therapy Centre, Finnish Red Cross Blood Service. The treatment regimens are presented in Figure 6.

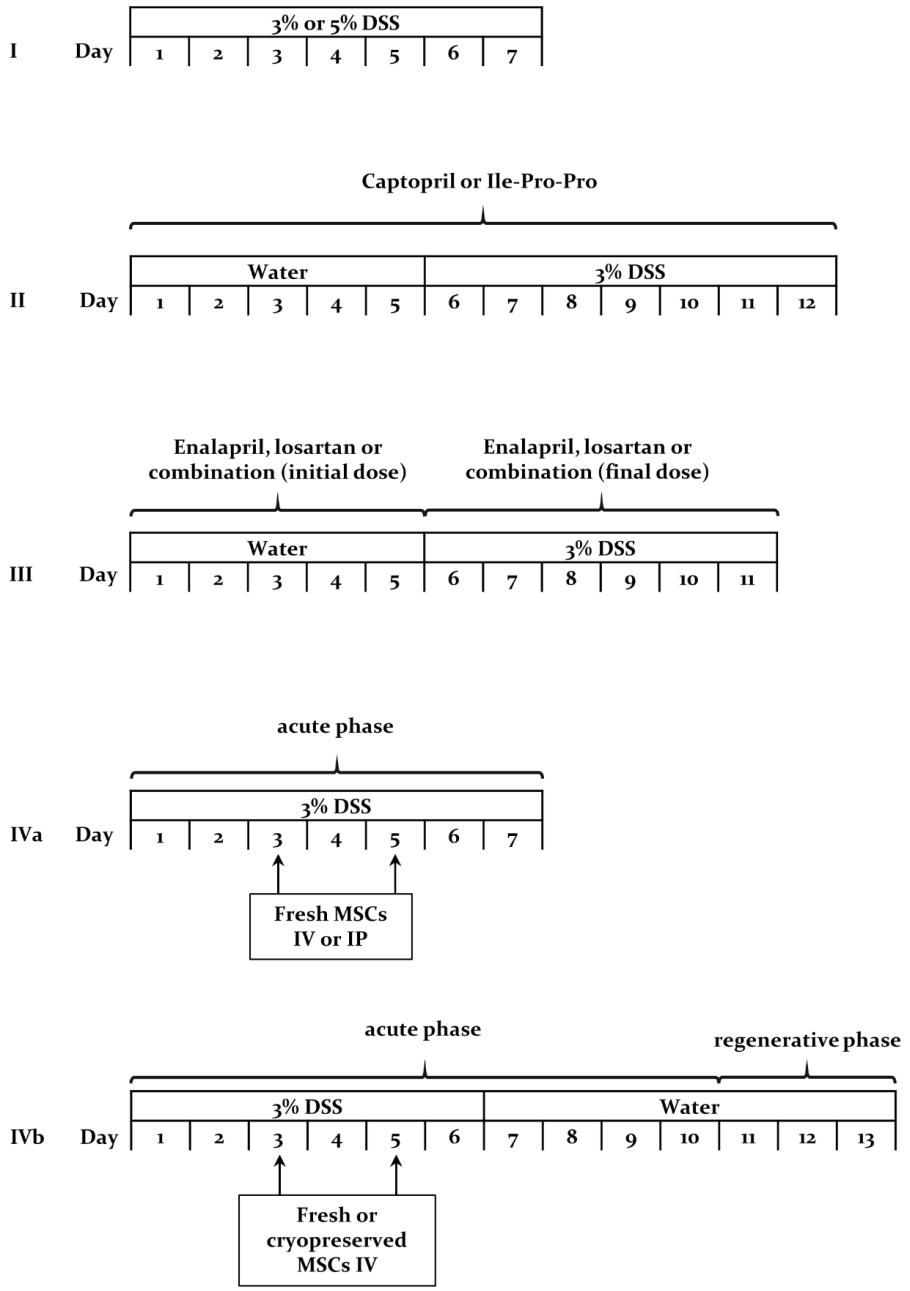

Figure 6. Study designs and timelines of each study. Fresh MSCs = freshly cultivated MSCs, IV = intravenously, IP = intraperitoneally.

4.3 Macroscopic assessment of inflammation

After sacrifice in Studies II, III and IV, colons were promptly excised and the lengths measured, and the typical manifestations of colitis were visually assessed. Stool consistency, tissue edema and visible blood were scored individually and summed as macroscopic scores. The scales were ranging from 0 to 2 in each category in Study II and III as follows: stool consistency (0 = normal, 1 = loose and 2 = liquid), bleeding and edema (0 = none present and 2 = clearly observable). In Study III, the scales were expanded so that stool consistency was scored on a scale of 0 - 4 (0 = normal, 1 = moist, 2 = loose, 3 = liquid, 4 = empty colon), and presence of blood (0 = none, 1 = streaks, 2 = clearly visible, 3 = heavy bleeding) and edema (0 = normal, 1 = mild, 2 = moderate and 3 = marked) on a scale of 0 - 3.

4.4 *Ex vivo*-incubation sample preparation

Intestinal incubation samples were prepared to quantify how much ACE was cleaved from intestinal samples and how much corticosterone was synthesized and secreted by intestinal epithelial cells. Sections of jejunum (Study I), ileum (Study III), proximal colon (Studies I, II and IV) or distal colon (Study III) were washed and incubated in pre-oxygenated Krebs buffer (119 mmol/l NaCl, 25 mmol/l $NaHCO_3$, 15 mmol/l KCl, 11 mmol/l Glucose, 1.6 mmol/l $CaCl_2$, 1.2 mmol/l KH_2PO_4, 1.2 mmol/l $MgSO_4$, chemicals obtained from Sigma-Aldrich) for 5-10 min at room temperature to minimize serum contamination, and then incubated in Krebs buffer for another 90 min at 37 °C in gentle agitation. The incubation supernatant was centrifuged clear of debris and used in immunochemical analyses. In Study I, Ang II or captopril (Sigma-Aldrich) in final concentrations ranging from 0.1 – 10 µmol/l for Ang II and 1 – 100 µmol/l for captopril were added into the incubation media to study stimulation and inhibition of corticosterone synthesis. Analyte concentrations were related to

the wet weight (Study I) or protein concentration of the corresponding tissue piece (Studies II, III and IV).

4.5 Histological assessment of inflammation

Histopathological changes were assessed qualitatively in Study I in jejunum and colon. In studies II, III and IV, the histopathological damage in colon was assessed quantitatively. Gradings of inflammatory cell infiltration and epithelial erosion were performed in Study II on a scale of 0 – 3, each, according to Erben et al., 2014 (Erben et al., 2014) and on a scale of 0 – 5 each in Study IV. In studies III and IV, the analysis of the samples was performed by an expert pathologist who was blinded to the treatment of the study groups. In Study III, a detailed analysis of mucosal injury (including sub-scores of surface epithelial injury, crypt epithelial injury, and crypt dilatation and distortion) as well as inflammation (including sub-scores for the presence of granulocytes, lymphocytes and macrophages, and granulomas) was executed and each were graded on a scale of 0 – 9.

4.6 Biochemical analyses

Protein and hormone analytes were quantified using commercial assays according to manufacturers' suggested protocols. ACE protein was measured in the *ex vivo*-incubation and tissue samples using Mouse ACE DuoSet ELISA (#DY1513, R&D System, Minneapolis, MN, USA) in all studies. Corticosterone concentration was measured in the *ex vivo*-incubation samples using Corticosterone EIA and ELISA kits (#500655 in Studies I, II and IV and #501320 in Study III, Cayman Chemical, Michigan, MI, USA). IL-1β and TNFα protein concentrations were quantified in colon tissue using AlphaLISA Detection Kits (AL503 and AL504, Perkin Elmer, Waltham, MA, USA) in Studies II and III.

Protein content of the corresponding tissue pieces were quantified using Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA).

4.7 Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

RT-qPCR was used in studies II, III and IV to measure the gene expression of selected members of intestinal RAS and glucocorticoid synthesis. Messenger RNA (mRNA) was extracted from mid-distal colon segments using NucleoSpin RNA (#740955, Macherey Nagel, Düren, Germany). Reverse-transcription of mRNA into complementary DNA (cDNA) was done using iScript cDNA Synthesis Kit (#1708891, BioRad, Hercules, CA, USA). All primers except for *Eef2* primers (Eissa et al., 2016) were designed using NCBI Primer Blast and ordered from Sigma-Aldrich (Sigma-Aldrich). RT-qPCR was run using LightCycler 480 SYBR Green I Master (Roche Diagnostics, Mannheim, Germany) and the LightCycler 480 equipment. Gene expression of target genes was normalized to the geometric mean of multiple reference genes *β-actin* and *S18* in Studies II and IV and *Rplp0*, *S18*, and *Eef2* in Study III using calculations originally presented in Hellemans et al., 2007 (Hellemans et al., 2007). The primer sequences used in this thesis are presented in Table 5.

4.8 Statistical analysis

Statistical analyses were conducted on either GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA) (Studies I and IV) or SPSS 22 and 23 (IBM, Armonk, NY, USA) (Studies II, III and IV). *p*-values lower than 0.05 were considered statistically significantly different.

The data were analyzed for normality using Shapiro-Wilk test and Levene's test for homogeneity of variances, when applicable, and parametric or non-

parametric tests were chosen accordingly. Logarithmic transformations were applied when possible in order to conduct parametric tests.

The differences between two groups were analyzed either with Student's unpaired *t*-test or Mann-Whitney U test. Differences between multiple groups were analyzed using one-way ANOVA followed by Tukey's (Studies III and IV) or Dunnet's (Study II) *post hoc* test or Kruskal-Wallis test followed by Mann-Whitney U test (Studies III and IV). Correlations were analyzed with either Pearson (Study I) or Spearman correlations (Study II). Weight development was analyzed using repeated measures ANOVA (Studies II, III and IV). *p*-values lower than 0.05 were considered statistically significant.

Table 5. A list of primer sequences for the genes and internal control genes analyzed in this thesis.

Gene	**Forward primer**	**Reverse primer**
Ace	5'-GCTGGAGGGTCTTTGATGGA-3'	5'-AGTCACCTTGGGATCTTGGC-3'
Agt	5'-CTTCCAAGGAACGATGAGAGGTT-3'	5'-ACAGACACCGAGATGCTGTT-3'
At1r	5'-CTGCTCTCCCGGACTTAACA -3'	5'-GCACTTGATCTGGTGATGGC-3'
Lrh-1	5'-GCATGGGAAGGAAGGGACAA-3'	5'-CGCTGATCGAACTGAAGGGA-3'
Cyp11b1	5'-GGAACCCACCATCAGTAAGGA-3'	5'-TCTTCCCTCACGCATGACAA-3'
Il-1β	5'-CTCCAGCCAAGCTTCCTTGT-3'	5'-TCATCACTGTCAAAAGGTGGCA-3'
β-actin	5'-CTGAATGGCCCAGGTCTGAG-3'	5'-AAGTCAGTGTACAGGCCAGC-3'
Rplp0	5'-TAACCCTGAAGTGCTCGACA-3'	5'-GGTACCCGATCTGCAGACA-3'
S18	5'-AACGAACGAGACTCTGGCAT-3'	5'-ACGCCACTTGTCCCTCTAAG-3'
Eef2	5'-TGTCAGTCATCGCCCATGTG-3'	5'-CATCCTTGCGAGTGTCAGTGA-3'

5 RESULTS

5.1 Interventions for treating experimental colitis

5.1.1 Assessment of colitis severity and resolution

The protocol for colitis induction was tested in study I using 3 and 5% DSS solution for 7 days. The animals fed and drank normally throughout the experiment and began manifesting signs of colitis, i.e. ruffled fur, diarrhea, and rectal bleeding, during the last few days of DSS administration. At the given regimens, colitis did not lead to hunched posture, an indication of serious pain, or lethality. In this study, 3% DSS did not disturb weight development of the mice, whereas 5% DSS caused weight loss and induction of *Il-1β* mRNA expression (unpublished data) in BALB/c mice (Figure 7). Induction of colitis was apparent with both DSS concentrations, due to marked and similar histopathological changes, including erosion, ulcerations, disrupted crypt structure, edema and inflammatory cell infiltrate (Figure 8).

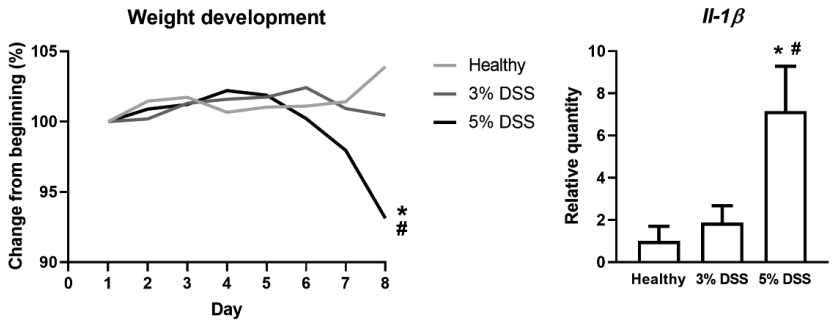

Figure 7. Weight development (n = 8 in each group) and colonic *Il-1β* mRNA expression (n = 5 in each group) indicating a more severe colitis in 5% DSS group. * indicates $p < 0.05$ compared to healthy and # indicates $p < 0.05$ compared to 3% DSS.

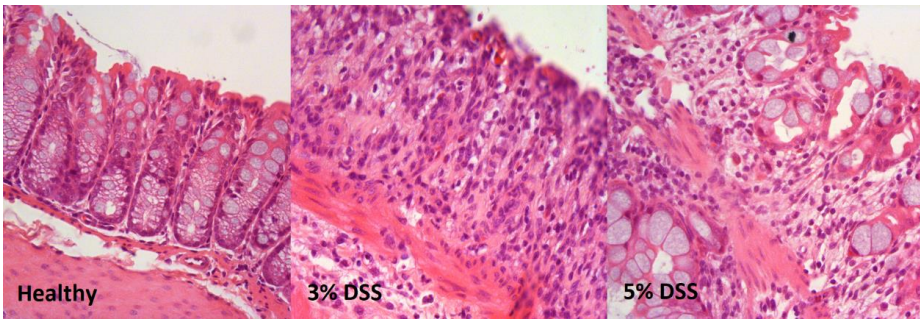

Figure 8. Hematoxylin and eosin stained slides show the disruption of the mucosal structures in DSS colitis.

Milder, 3% DSS-induced colitis was chosen for Studies II, III and IV to investigate interventions that could potentially alleviate colitis and modulate glucocorticoid synthesis. Colitis severity and its resolution was assessed by measuring the body weights of the animals and lengths of colons, grading macroscopic and histopathological inflammation, and quantifying protein or mRNA levels of IL-1β and TNFα. Macroscopic and histopathologic scores of colitis groups were consistently increased by DSS in all studies (Table 6). The histopathological changes in acute colitis included loss or deformation of crypts, erosion, ulceration, mucosal and submucosal inflammatory cell infiltrate, and appearance of granulomatous foci. Distinct surface and crypt epithelial cell hyperplasia were seen in regenerative phase of colitis (Study IVb). Macroscopic changes consisting of stool consistency, colonic edema and bleeding were present but in a varying degree. Colons of mice were shortened by DSS administration in Studies II and III. The weights of the mice developed somewhat differently between the studies even under the same colitis protocols. The weights of the animals receiving 3% DSS declined significantly in Study II and Study IVb but were not statistically significantly different compared to healthy controls in Study I, III and IVa. Proinflammatory cytokines, IL-1β and TNFα were prominently induced by DSS in Studies II and IV, and to a lesser degree in Study III. Induction of colitis was evident in each

study based on histopathological damage, macroscopic manifestations and proinflammatory cytokine levels.

5.1.2 Enalapril and losartan alleviate colitis and reduce inflammatory infiltrate in *lamina propria*

Both an ACE inhibitor enalapril and an angiotensin II receptor blocker losartan alleviated colitis in Study III at doses of 3 and 10 mg/kg/d, respectively, by reducing inflammatory cell infiltrate and granulomatous foci in *lamina propria* of colon, which together accounted for the inflammation score (Table 6). Losartan, in addition, reduced macroscopic scores. The reduction could not be accounted for by any of the individual parameters (stool consistency, edema, bleeding), but rather the overall impression was milder in losartan group compared to DSS group. Enalapril completely suppressed the induction of colonic *Il*-1β mRNA expression (Figure 9). The combination of enalapril and losartan at the prementioned doses, or ACE inhibitor captopril at a dose of 5 mg/kg/d or Ile-Pro-Pro at a dose of 227 mg/kg/d in Study II, did not improve colitis by the measured parameters.

5.1.3 Xenogeneic mesenchymal stromal cells have a limited anti-inflammatory effect on colitis

Mesenchymal stromal cell treatment was examined at two different timepoints of the inflammation process in Study IV (Figure 6). In acute phase of colitis in Study IVa, intravenously or intraperitoneally administered freshly cultivated MSCs did not improve body weight loss or macroscopic inflammation. Colonic IL-1β and TNFα protein levels were increased in mice receiving 3% DSS and vehicle for 7 days, and the increase of IL-1β was completely abolished by intravenously administered MSCs, indicating a slight

benefit (Figure 9). In the next part of the study (Study IVb) (Figure 6), body weight first declined in all colitis groups and begun to recover from day 10 to 11 onwards. At the end of the experiment, colitis was still evident in colitis groups based on lower bodyweights, increased macroscopic and histopathology scores, shortening and increase of weight of colons relative to the body weight. Colonic IL-1β and TNFα protein and *Il-1β* mRNA levels were increased in mice receiving either vehicle or cryopreserved MSCs, but the increase of proinflammatory cytokines did not reach statistical significance in the group receiving freshly cultivated MSCs (Figure 9) Neither freshly cultivated nor cryopreserved MSCs improved body weight development, macroscopic scores, colon length or weight, histopathological damage, or levels of proinflammatory cytokines.

Table 6. Colitis severity in different studies. Colon lengths are presented as mean ± standard deviation and disease scores as median (Interquartile range). † $p < 0.05$ compared to healthy, * $p < 0.05$ and ** $p < 0.01$ compared to control. IVa acute and IVb regenerative phases of colitis, IP = intraperitoneally, IV = Intravenously. n = 8 – 10 in each group.

Study	Treatment	Colon length (cm)	Macroscopic score	Histopathology score	Injury score	Inflammation score
II	Healthy	9.3 ± 0.3	0.0 (0.0)	0.0 (0.0)		
	DSS	6.5 ± 0.5 †	3.5 (3.3) †	4.0 (3.0) †		
	DSS + Captopril	6.0 ± 0.5 †	4.0 (2.0) †	4.0 (1.8) †		
	DSS + Ile-Pro-Pro	5.6 ± 0.6 *†	4.0 (2.5) †	5.0 (1.8) †		
III	Healthy	7.7 ± 0.9	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	DSS	6.7 ± 0.6	5.0 (4.8) †	10.5 (6.5) †	5.5 (6.0) †	5.0 (1.5) †
	DSS + Enalapril	7.2 ± 0.7	4.5 (5.5) †	6.0 (5.8) *†	4.0 (3.5) †	2.0 (2.8) **†
	DSS + Losartan	7.0 ± 1.1	2.5 (4.3) *†	6.5 (8.1) †	4.0 (5.3) †	2.8 (2.8) *†
	DSS + Enalapril + Losartan	7.2 ± 0.5	5.0 (5.3) †	6.5 (8.3) †	4.0 (3.5) †	3.0 (4.8) †
IVa	Healthy	7.2 ± 0.3	0.0 (0.0)			
	DSS + VE IV	5.5 ± 0.2 †	2.5 (4.3)			
	DSS + MSC IV	5.3 ± 0.1 †	3.0 (2.8) †			
	DSS + VE IP	4.9 ± 0.2 †	4.0 (2.0) †			
	DSS + MSC IP	4.9 ± 0.2 †	4.0 (1.0) †			
IVb	Healthy	8.1 ± 0.3	0.0 (0.0)	0.0 (0.0)		
	DSS + VE	6.1 ± 0.3 †	2.0 (1.1) †	5.8 (2.5) †		
	DSS + Fresh MSCs	6.5 ± 0.3 †	2.0 (2.0) †	5.0 (2.5) †		
	DSS + Cryopreserved MSCs	6.5 ± 0.5 †	2.0 (2.5) †	7.0 (6.5) †		

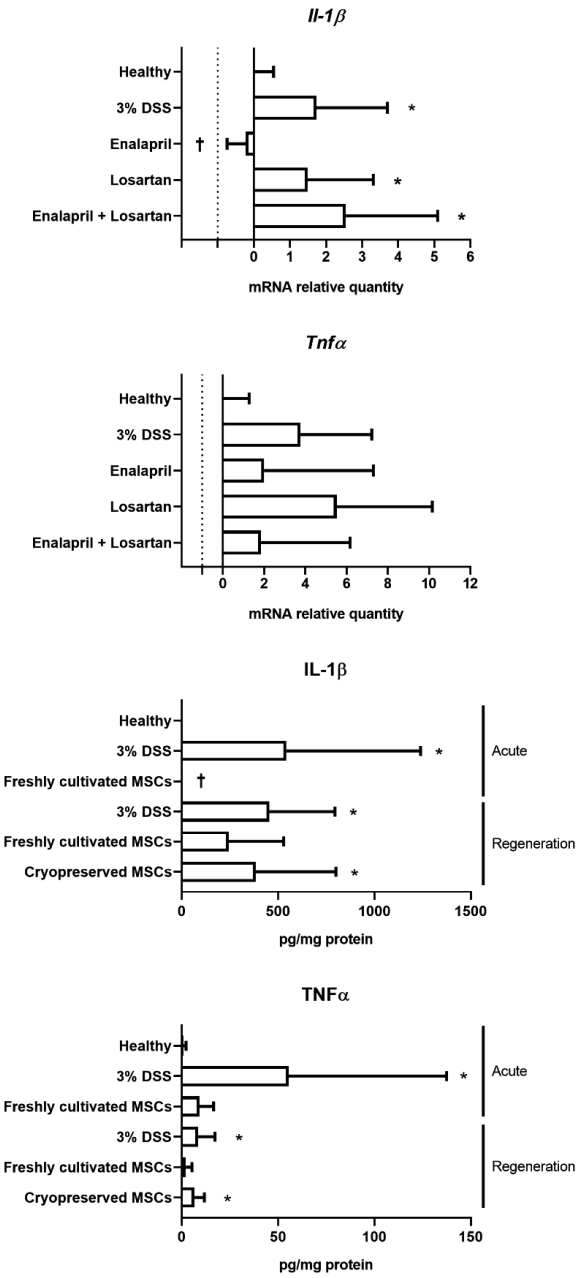

Figure 9. Proinflammatory cytokines. mRNA expression of *Il-1β* and *Tnfα* (two upper figures) in Study III, and protein levels (two lower figures) in Study IV. Values are relative to healthy mice. Asterix indicates $p < 0.05$ compared to healthy and cross $p < 0.05$ compared to 3% DSS group. n = 4 – 10 in each group.

5.2 Renin-angiotensin system in experimental colitis

5.2.1 ACE shedding is induced by inflammation and is location dependent

Basal shedding of ACE was readily detected in incubation samples of all studied parts of the intestine, which were proximal colon in Studies I, II and IVb and distal colon in Study III, jejunum in Study I, and ileum in Study III (unpublished data) (Figure 10). However, whether ACE shedding was induced by inflammation, was dependent on the concentration of DSS and localization in colon. An increase in ACE shedding was induced by 3% DSS colitis in distal colon (Study III). In proximal colon, 5% DSS administration caused a clear increase in ACE shedding, whereas the shedding was not affected by 3% DSS administration (Figure 10). In small intestine, 3% DSS induced an increase in ACE shedding in jejunum (Study I) but not in ileum (Study III). Differences in ACE shedding were not reflected in ACE tissue concentrations or gene expression (Figure 10 and 11), but they were quantified in mid and mid-distal colon, respectively.

5.2.2 Ile-Pro-Pro and cryopreserved MSCs reduce ACE shedding

ACE shedding was reduced compared to untreated animals in proximal colon by Ile-Pro-Pro (Study II) and cryopreserved MSCs (Study IV) (Figure 10), while the reduction by captopril or freshly cultivated MSCs showed only a tendency for lower values. Cryopreserved MSCs also reduced the tissue level of ACE protein, possibly indicating a feedback mechanism to reduce proinflammatory Ang II signaling. The reduction in ACE shedding could not be explained by changes in the gene expression of *Ace* as it was not changed by Ile-Pro-Pro or cryopreserved MSCs (Figure 11).

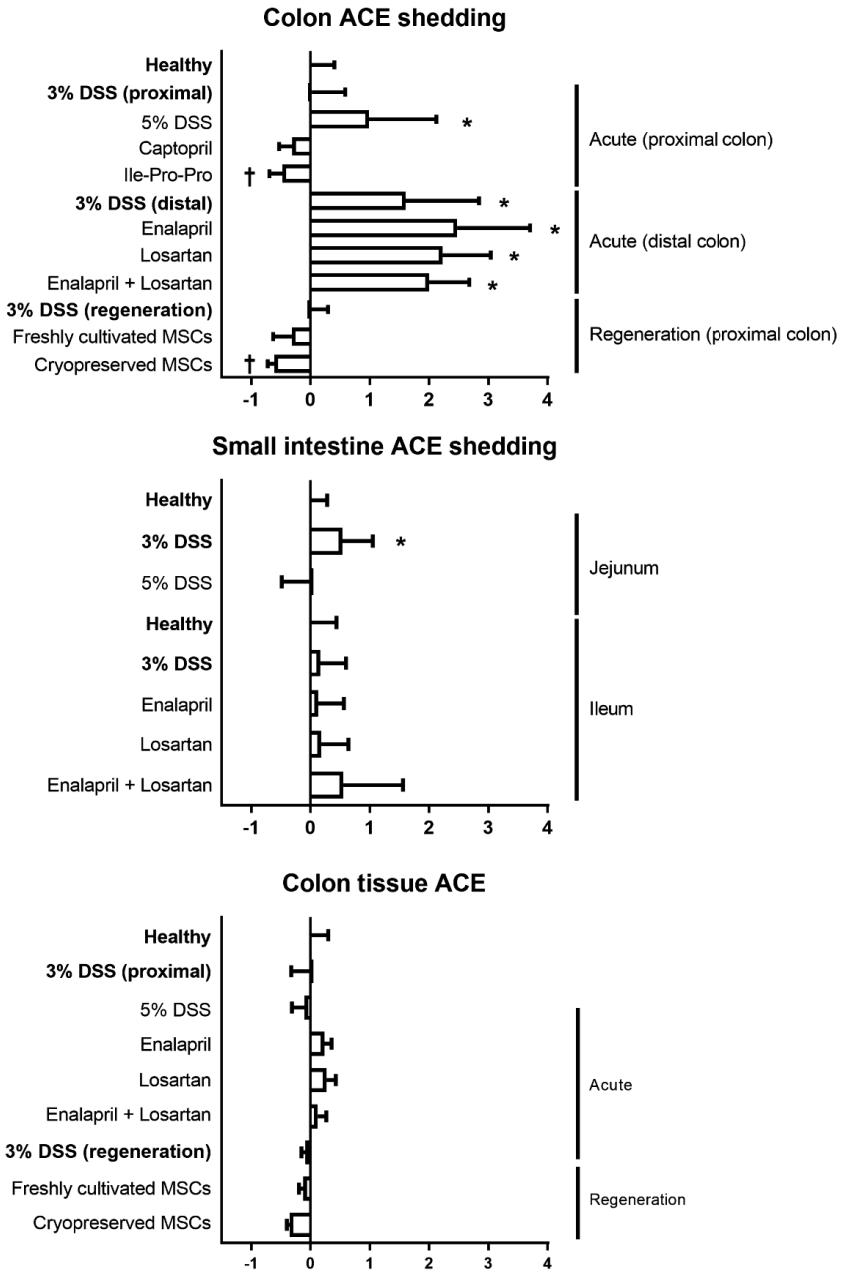

Figure 10. ACE protein shedding in colon (upper panel), small intestine (middle panel), and tissue levels in colon (lower panel) in different studies. Values are relative to healthy mice. Asterix indicates $p < 0.05$ compared to healthy and crosses $p < 0.05$ compared to 3% DSS group. n = 6 – 10 in each group.

5.2.3 Gene expression of renin-angiotensin system components is not influenced by acute colitis

The colonic mRNA expression of angiotensinogen (*Agt*), *Ace*, *At1r* and *At2r* was compared between acute colitis and healthy intestine (Studies I, II and IV) and during the healing process (Study IVb, partly unpublished data). In general, genes of RAS components were expressed at similar levels in healthy and inflamed intestine in acute colitis, but the expression of *Agt* seemed to be increased (p = 0.071) in mice receiving 5% DSS (unpublished data, Study I) (Figure 11) During the regenerative phase, *At1r* expression was downregulated and *Lrh-1* expression only seemed to be reduced.

5.2.4 Captopril decreases colonic gene expression of *Ace* and *Agt*

The effect of captopril (Study II), enalapril, losartan (Study III) and the ACE-inhibiting peptide Ile-Pro-Pro (Study II) on mRNA expression of RAS components (*Agt*, *Ace*, *At1r*) was examined parallel to the resolution of colitis. Although expression of *Ace* or *Agt* were not induced by colitis, their expression was reduced by captopril compared to DSS group (Figure 11). Similar effect was not seen with enalapril, losartan or Ile-Pro-Pro, indicating that captopril could have additional mechanisms than ACE inhibition in colon.

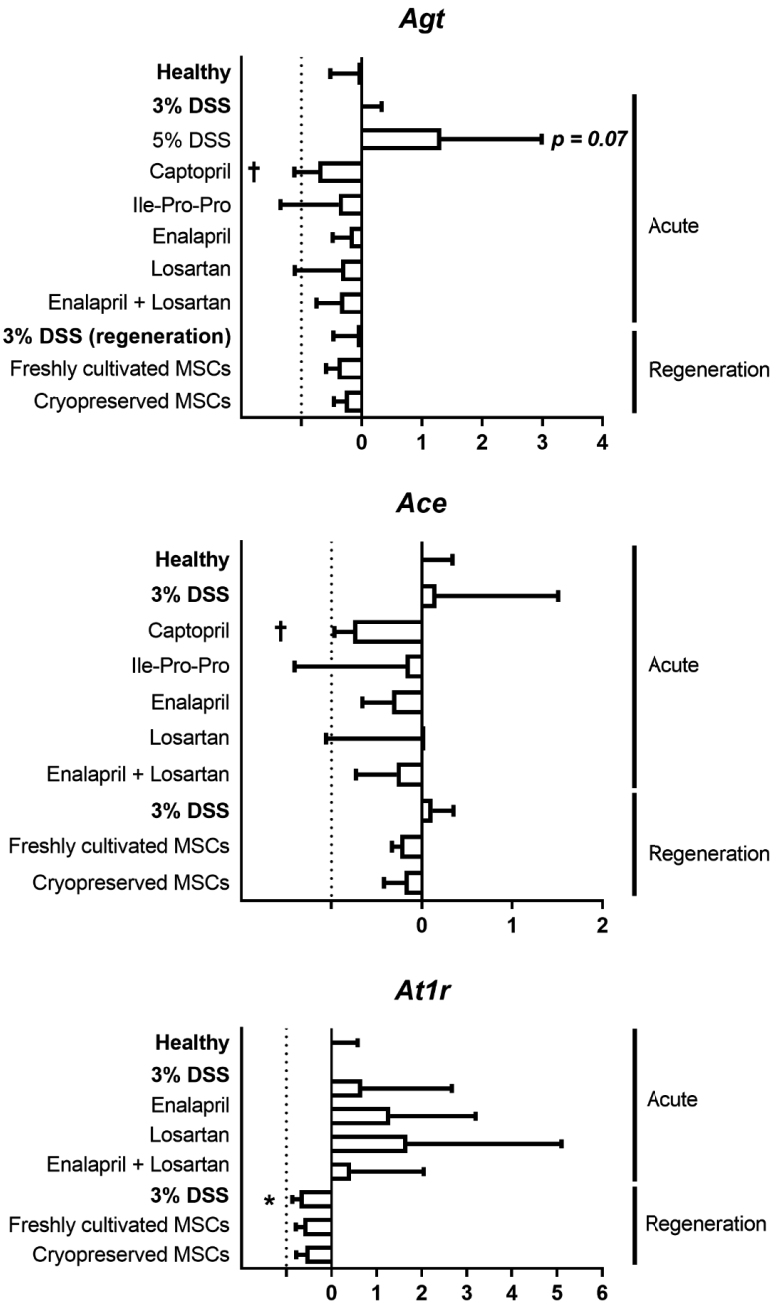

Figure 11. Gene expression of the measured RAS components. Values are relative to healthy mice. Asterix indicates $p < 0.05$ compared to healthy and cross $p < 0.05$ compared to 3% DSS group. n = 5 – 7 in each group.

5.3 Glucocorticoid synthesis in gastrointestinal tract

5.3.1 Colitis induces corticosterone synthesis in proximal colon

The synthesis of corticosterone in tissue incubations was measured in each study. Like with ACE shedding, DSS-induced corticosterone synthesis was dependent on the studied section of colon. Corticosterone synthesis was induced in acute colitis by 3% and 5% DSS in proximal colon in Studies I and II (Figure 12), parallel to weight loss of the mice (Study II) and colon shortening. On the contrary, corticosterone synthesis was not increased by 3% DSS in distal colon in Study III, but at the same time, normal weight development and colon lengths indicated an overall milder colitis. When measured during the healing process in Study IVb, although lower weight and colon shortening indicated an ongoing inflammation, corticosterone production in proximal colon was not increased in mice treated with 3% DSS either. The changes in corticosterone synthesis were not reflected in the transcription factor *Lrh-1* or the rate-limiting enzyme *Cyp11b1* mRNA expression, which were measured in mid-distal colon.

5.3.2 Angiotensin II induces corticosterone synthesis *ex vivo*

Stimulation and inhibition of corticosterone synthesis was tested *in vitro* in Study I, using *ex vivo* small intestine incubations. Ang II induced corticosterone synthesis at the highest tested concentration of 10 μM in the incubation media, and the induction appeared dose dependent (Figure 12). Captopril alone did not inhibit basal corticosterone synthesis at tested concentrations.

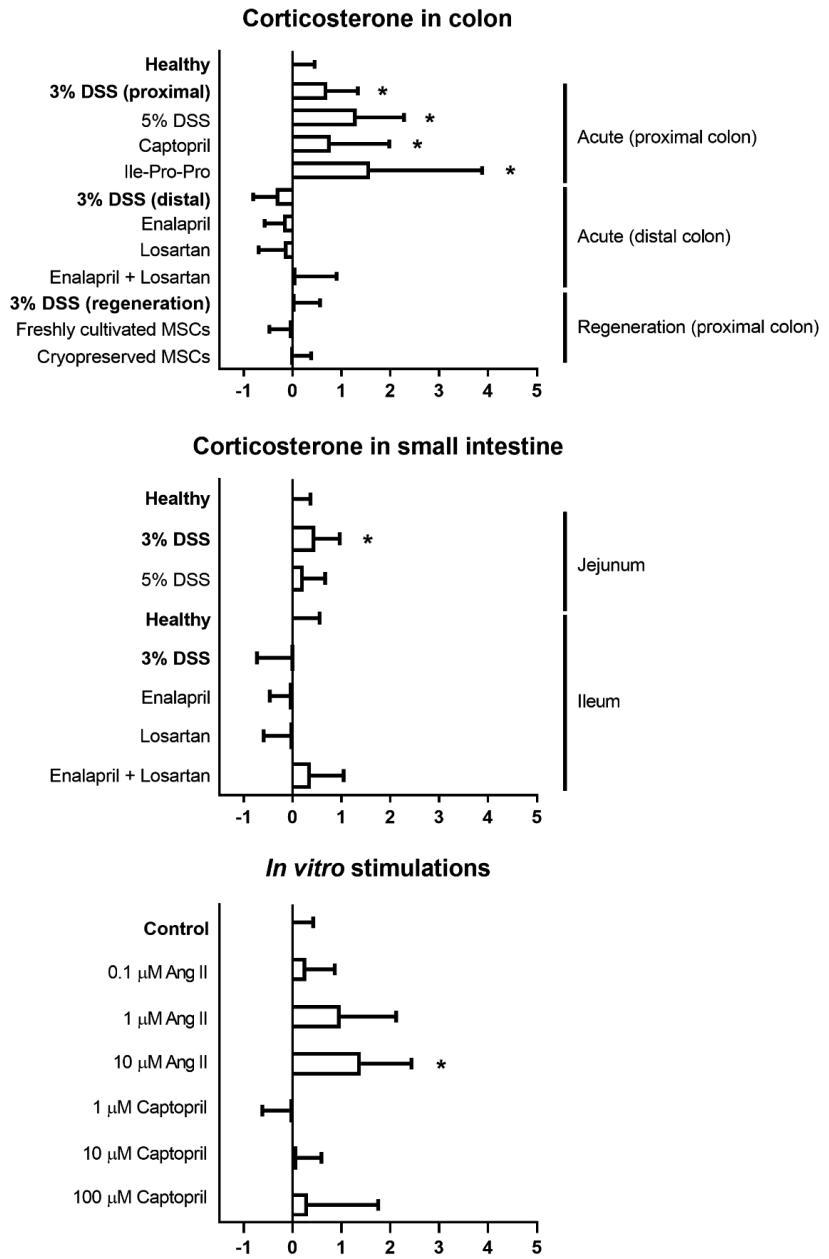

Figure 12. Corticosterone production in different parts of colon (upper panel) and small intestine (middle panel) in different studies (n = 6 – 10 in each group), and in jejunum in the presence of different concentrations of Ang II or captopril (lower panel) (n = 5). Values are relative to healthy mice. Asterix indicates $p < 0.05$ compared to healthy group.

5.3.3 Inhibition of RAS *in vivo* does not affect corticosterone synthesis

The ability of RAS inhibitors to modulate corticosterone synthesis during established inflammation was examined *in vivo* using ACE inhibitors captopril (Study II) and enalapril (Study III), angiotensin II receptor blocker losartan (Study III) and the ACE inhibiting tripeptide Ile-Pro-Pro (Study II). Captopril completely suppressed the gene expression of *Cyp11b1* (Figure 13), but it did not affect corticosterone production *ex vivo* in colon incubations (Figure 12). Enalapril, losartan or Ile-Pro-Pro did not have a similar effect on *Cyp11b1* expression. None of the compounds inhibited corticosterone synthesis *in vivo*, despite the suppression of *Cyp11b1* by captopril. *Lrh-1* gene expression was similar in healthy and inflamed intestine.

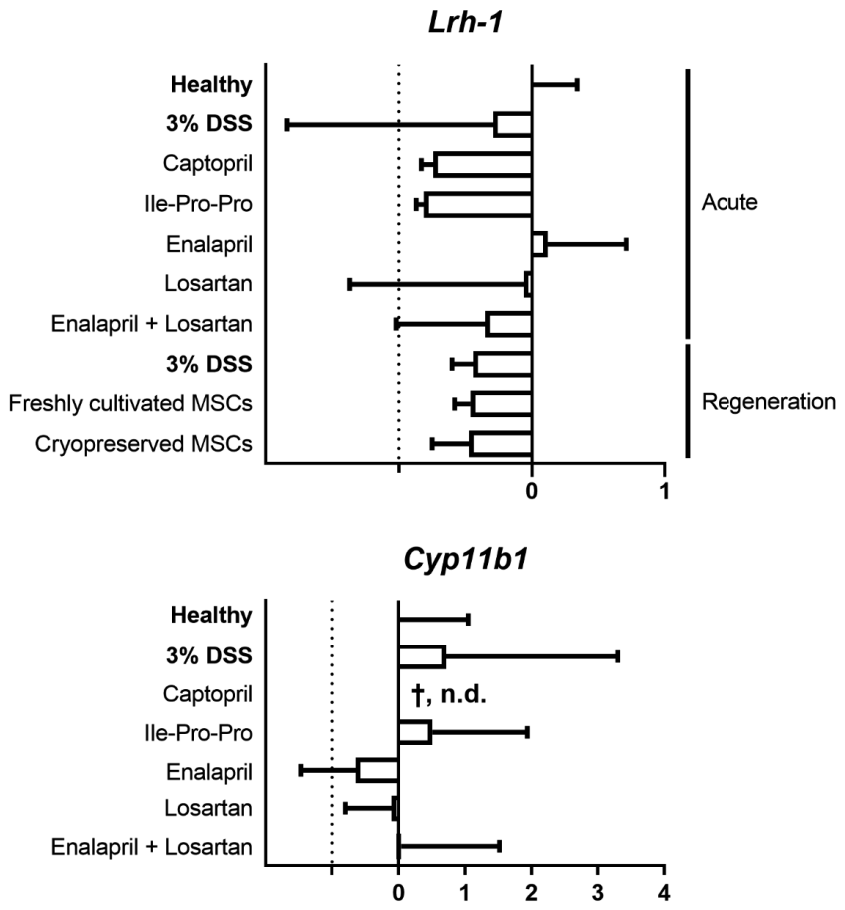

Figure 13. Gene expression of *Lrh-1* and *Cyp11b1*. Values are relative to healthy mice. Cross indicates $p < 0.05$ compared to 3% DSS group. n = 3 – 7 in each group.

6 DISCUSSION

6.1 Interventions for treating experimental colitis

This series of studies investigated treatments for intestinal inflammation in the mouse DSS model in BALB/c mice, with a focus on local intestinal renin-angiotensin system. A mild to moderate severity colitis in BALB/c mice using 3% DSS administration was chosen to avoid obscuring the therapeutic effects of the interventions by overly severe colitis. The study designs were devised to measure the magnitude of changes caused by colitis and to compare the effects of interventions on these parameters during colitis, but the effects of these interventions to healthy intestine are unknown. Histopathological changes and cytokine profile in DSS colitis share similarities with human ulcerative colitis (Chassaing et al., 2014; Perše and Cerar, 2012). Although they represent only a part of pathogenesis of human IBD, treatments for IBD also alleviate DSS colitis, making the model relevant for investigating treatments for intestinal inflammation (Melgar et al., 2008). BALB/c mice are more resistant to DSS colitis than the commonly used C57BL/6 strain (Melgar et al., 2005), but their propensity for colitis resolution and Th2/Th17-polarized inflammation resembling ulcerative colitis make BALB/c mice suitable for studying interventions (Yang et al., 2017).

6.1.1 The potential of RAS modulation as a treatment for intestinal inflammation

The ability of RAS inhibition to prevent and alleviate colitis was tested in Study III by administering ACE inhibitor enalapril, angiotensin II receptor blocker losartan or their combination in drinking fluid along with 3% DSS. The results demonstrate, that oral administration of RAS inhibitors can be feasible and effective in prevention of experimental colitis. Of the drugs

tested, enalapril had the strongest anti-inflammatory effects at tested doses regarding improvement of histopathology and proinflammatory cytokine levels. Losartan improved histopathological inflammation and macroscopic manifestations of colitis without improving proinflammatory cytokine levels. This could mean, that at larger doses, losartan might be more efficient than enalapril at alleviating colitis. The results are in agreement with previous studies employing oral administration of enalapril and losartan at similar doses in murine DSS and TNBS colitis models, with findings that both drugs alleviated histopathological damage and losartan improved macroscopic inflammation (Lee et al., 2014; Liu et al., 2016). Captopril, in our hands, had no therapeutic effect, which might be due to both the low dose (5 mg/kg/d) and the more potent induction of colitis, although this is not completely consistent with previous studies reporting alleviation of colitis on rats on captopril doses as low as 0.1 mg/kg/d (Jahovic et al., 2005). Higher, 50 mg/kg/d doses have also been beneficial (Wengrower et al., 2004).

Captopril, enalapril and losartan are antihypertensive drugs and although we did not measure blood pressure of the animals, there are reports that similar doses that were used in our studies do not induce hypotension for normotensive animals (Diop-Frimpong et al., 2011; Harding et al., 1993). The doses used in preclinical studies by us and others (Jahovic et al., 2005; Lee et al., 2014; Liu et al., 2016) do not represent clinical dosing for human hypertension, but are larger than those, owing to the nature of the common colitis models which employ a severe short-term inflammation. Better approximation of effective doses and comparison to existing medications could be achieved in chronic colitis models, like the recurring low-dose DSS model. The active metabolites of enalapril and losartan have short half-lives, at least in humans, and administration of drugs in drinking fluid served to avoid repeated gavaging two to three times per day to ensure stable levels of drugs in the body throughout the day. Oral administration of RAS inhibitors in drinking fluid has been reported with losartan in TNBS model (Shi et al., 2016) and valsartan in TNBS and DSS models (Santiago et al., 2008), but

enalapril has not been administered in drinking fluid in experimental colitis. We could show that co-administration of enalapril and losartan with DSS were effective in alleviating colitis.

The new findings in these studies are that, firstly, the alleviation of colitis was specifically associated with reduction of inflammatory infiltrate, and secondly, combination treatment with the effective doses of losartan and enalapril were not beneficial nor synergistic in treating colitis. Reducing Ang II formation by other enzymes, like chymase, and simultaneous blocking of Ang II signaling should lead to effective inhibition of AT1R-mediated proinflammatory actions, and theoretically, more potent alleviation of colitis. Since this was not the case, the results imply that combination treatment does not bring added benefits over single medications in treatment of intestinal inflammation, but the underlying reason for the loss of benefit in combination treatment remained unsolved. For treatments with single drugs, the mechanism behind reduced inflammatory infiltrate could be due to inhibition of leukocyte homing. AT1R regulates expression of MAdCAM-1, an adhesion molecule, which is induced by intestinal inflammation and IBD (Briskin et al., 1997; Mizushima et al., 2010). MAdCAM-1 increases homing of T cells to inflamed intestine by binding to α4β7-integrin at T-cell surface (Berlin et al., 1993; Briskin et al., 1997). The role of intestinal RAS in leukocyte homing has been investigated in one study, which found that candesartan downregulated Ang II -mediated upregulation of MAdCAM-1 in an endothelial cell lines and AT1R knockout mice expressed MAdCAM-1 at lower quantities during colitis (Mizushima et al., 2010), but the effect of RAS inhibitors on MAdCAM-1 expression has not been studied in wildtype animals. The relevance of downregulation of these adhesion molecules and reducing leukocyte attraction by RAS inhibitors in intestinal inflammation is highlighted by that antibodies blocking α4β7-integrin, vedolizumab, and the α4-subunit, natalizumab, are approved for treatment in IBD and MAdCAM-1 antibodies are under development (Duijvestein and D'Haens, 2019). A more general mechanism of alleviating inflammation in our study, and in line with the

downregulation of *Il-1β* mRNA expression, might be the actions of RAS inhibitors on proinflammatory leukocytes, which have been shown to increase production of proinflammatory cytokines in response to Ang II and inhibit their production in response to RAS inhibitors (Fukuzawa et al., 1997; Lapteva et al., 2002).

6.1.2 Non-activated mesenchymal stromal cells a potential treatment for intestinal inflammation

The feasibility and safety of human bone-marrow derived platelet-lysate expanded MSCs were examined in intestinal inflammation, along with comparison of administration routes, and freshly cultivated and cryopreserved MSC. The results suggest a mild anti-inflammatory effect of non-activated xenogeneic freshly cultivated MSCs in terms of reduced proinflammatory cytokine levels, but they were not able to improve histopathological damage or prevent weight loss. Non-activated human MSCs have not been as efficient as pre-activated MSCs in treating mouse DSS and TNBS colitis (Duijvestein et al., 2011; Fan et al., 2012; Kim et al., 2013), which is likely caused by incomplete reactivity between mouse and human cytokines and their receptors leading to insufficient MSC activation in mice (Chen et al., 2009; Galleu et al., 2017; Zachar et al., 2016). IFN-γ, specifically, is crucial to the development of DSS colitis (Ito et al., 2006) and an important inducer of MSCs' anti-inflammatory functions but lacks species cross-reactivity (Bernardo and Fibbe, 2013). However, since cell therapies in clinical applications utilize non-activated MSCs, avoiding additional manipulation of MSCs is still appropriate in preclinical testing. Some activation of MSCs in our xenogeneic model could possibly have been achieved by other universal mechanisms like nitric oxide (Chen et al., 2015; Kankuri et al., 1999), hypoxia, and activation of Toll-like receptors by for example lipopolysaccharide (LPS) (Crisostomo et al., 2008;

Krampera, 2011; Najar et al., 2017), explaining the limited beneficial effect of MSCs.

The cryopreserved MSC product used in our study differs from many MSC products used in clinical studies of IBD (Gregoire et al., 2017) in that it has been cultivated in human platelet lysate instead of fetal bovine serum, eliminating xenogeneic agents from the final product. It has already been used in clinical applications like treating graft-versus-host disease, with good response rate to the treatment (Salmenniemi et al., 2017), and our data indicates feasibility of use in treatment of intestinal inflammation. Although the beneficial effects were gained using freshly cultivated MSCs, the data does not support conclusions between the efficacy of freshly cultivated and cryopreserved MSCs, due to the overall mild therapeutic effect. Since beginning of this work, reports have been cautiously positive on the efficacy of non-activated MSCs in clinical trials treating human IBD (Dave et al., 2015; Gregoire et al., 2017; Qiu et al., 2017), which encourages continuing investigations these MSCs in clinical studies of IBD, as well.

We found that MSCs downregulate colonic ACE and subsequently its shedding, which could be taken as a beneficial response to the anti-inflammatory actions of MSCs as Ang II induces NF-κB in various tissues (Suzuki et al., 2003). On the other hand, activation of NF-κB induces ACE and AT1R expression (Nishida et al., 2011; Shorning et al., 2012), so downregulation of ACE might be a consequence of reduced NF-κB activation, although this would need to be verified. Recent studies have shown, that microvesicles from MSCs downregulate *Ace* and upregulate *Ace2* gene expression, consequently lowering Ang II to Ang 1-7 ratio, in a model of pulmonary arterial hypertension (Liu et al., 2018), and that MSCs downregulate ACE protein expression in a renovascular hypertension model in kidney (Lira et al., 2017; Oliveira-Sales et al., 2013). As far as we know, we are the first to show that MSCs downregulate ACE in intestine.

6.2 Renin-angiotensin system in intestinal inflammation

6.2.1 ACE shedding is increased by inflammation

This series of studies demonstrate, for the first time in intestine, a specific induction of ACE protein solubilization, ACE shedding, by inflammation. The induction of ACE shedding arguably parallels at least moderate inflammation, as DSS colitis has a strong tendency for increasing damage of intestinal mucosa from proximal towards distal colon. This would explain why ACE shedding was induced only in distal colon in 3% DSS-induced colitis, whereas the shedding by 5% DSS was induced in more proximal segment of colon as well. We also saw an increase in shedding in jejunum by 3% DSS but the reason for lack of dose-dependency of DSS is unsolved. Shedding of ACE in intestine by inflammation is in line with studies reporting increased plasma ACE concentrations in sepsis and lung injury in human patients (Orfanos et al., 2000; Votta-Velis et al., 2007), suggesting that ACE shedding is a universal mechanism in inflammatory conditions.

The origin of solubilized ACE in intestine is unknown. In healthy colon, ACE is localized in vascular endothelium and to a degree in epithelium, but many immune cells carry ACE protein at their surface (Chen et al., 2013b; Danilov et al., 2003). There are reports of elevated ACE protein expression in pathological conditions, including DSS colitis (Spencer et al., 2007) and in human colorectal carcinoma, where ACE was localized at the apical surface of epithelial cells (Rocken et al., 2007), and in intraepithelial lymphocytes in a model of short bowel syndrome (Wildhaber et al., 2005). We could not corroborate the increase or depletion of ACE, either on protein or mRNA level, in mild to moderate DSS-colitis.

Many surface proteins, like cytokines, are cleaved from cell membranes to elicit their paracrine effects, but virtually nothing is known about the

physiological function of ACE shedding. So far it is known that basal and induced shedding of ACE are differentially regulated and executed (Balyasnikova et al., 2002; Kohlstedt et al., 2004; Kohlstedt et al., 2002), and now we show that colitis increases ACE shedding in colon. Intuitively, the consequence could be that by shedding ACE, the proinflammatory Ang II production is reduced locally in the colonic mucosa, although the fact that ACE remains active post-cleavage indicates additional functions, like perhaps increasing the concentration of local Ang II in tissues where the soluble ACE is transferred. Alternatively, as retention of ACE at the cellular membrane is associated with activation of ACE signaling (Fleming, 2006), increased ACE shedding could be a way to downregulate its signaling function.

6.2.2 RAS genes are not induced in mild to moderate colitis

The genes of RAS or tissue-bound ACE protein were not induced by acute colitis, which is in contradiction with reports of increased ACE and AT1R protein levels and *At1r* mRNA expression in colonic epithelium in DSS model (Katada et al., 2008; Okawada et al., 2016; Spencer et al., 2007). The differences might be explained by the relatively mild colitis in our series of studies, or by strain differences, as all the aforementioned studies were conducted using C57BL/6 mice, which are more susceptible to DSS colitis (Melgar et al., 2005). On the contrary, during the healing process of colitis, our results show a specific downregulation of *At1r* gene expression, which could be assumed to be a feedback mechanism to inflammation and elevation of Ang II.

6.2.3 ACE inhibitors are not alike

We found that, unlike enalapril, losartan, or Ile-Pro-Pro, captopril downregulated *Ace, Agt* and *Cyp11b1* expression, implying that captopril has additional mechanisms of action than merely ACE inhibition in the intestine.

This is in agreement with a report that captopril can downregulate *ACE* gene expression in human dendritic cells (Lapteva et al., 2002), but contradicts with findings that other ACE inhibitors, ramiprilat and perindoprilat, increase *Ace* expression *in vitro* and *in vivo* in vascular endothelial cells (Kohlstedt et al., 2004). The reduction of *Ace* expression in our study was not fully reflected in ACE shedding, which was not significantly reduced in colon.

Effects of Ile-Pro-Pro on gene and protein expression were tested alongside captopril, and their effects on ACE protein and gene expression were different. Ile-Pro-Pro reduced ACE shedding without affecting the gene expression of *Ace*, *Agt* or *Cyp11b1*, supporting the assumption that RAS inhibiting compounds have different modes of action. The reduction of ACE shedding by Ile-Pro-Pro is a novel finding, but due to the lack of available comparison, as blood pressure studies using Ile-Pro-Pro generally have not reported plasma ACE concentrations, the mechanism can only be speculated. It could be related to locking ACE to a more stable conformation or be mediated by other enzymes and receptors entirely, but reduced ACE protein expression cannot be excluded based on our data. Ile-Pro-Pro can modulate activity of other enzymes, like activate cathepsin G, which forms Ang II from Ang I (Klickstein et al., 1982), and inhibit prolyl oligopeptidase, which forms Ang 1-7 from Ang I and Ang II (Welches et al., 1993), and arginase at high concentrations (Lehtinen et al., 2010; Siltari et al., 2012), thus confounding its effect on RAS *in vivo*, but offer no explanation on the results of ACE shedding. Ile-Pro-Pro shares structural similarities with the natural activator of ACE signaling, bradykinin, also capable of retaining ACE on the cell membrane (Kohlstedt et al., 2002), in that they both contain a double-proline motif. Ile-Pro-Pro might retain ACE on the cell surface by activation of ACE signaling, which was reported also in the case of ACE inhibitors ramiprilat and perindoprilat (Kohlstedt et al., 2004), but without data on ACE phosphorylation status, this assumption remains speculative. Whether the effects of captopril and Ile-Pro-Pro on intestinal RAS are specific to inflammatory conditions or if they apply to healthy intestine as well, cannot be concluded based on our results.

Contrary to the effects of captopril and Ile-Pro-Pro, enalapril had no effect on ACE expression or shedding, which further highlights the differences between ACE inhibitors.

6.3 Glucocorticoids of the intestine

Acute inflammation in the intestine induces colonic synthesis of anti-inflammatory glucocorticoids (Coste et al., 2007). Since RAS activation is involved in intestinal inflammation, we wanted to study the involvement of RAS in glucocorticoid production in the intestine. Corticosterone synthesis was measured as the release of corticosterone from *ex vivo* incubated intestinal samples during a time course which was tested to yield differences between healthy and colitic mice (90 minutes) before reaching the full capacity of synthesis (at 3 hours) to avoid confounding effects of tissue degradation. As steroid hormones are lipophilic, they cannot be stored inside the cells, but are synthesized at demand, and therefore, the measured corticosterone in the sample can be assumed to be produced *in situ*.

6.3.1 Induction of glucocorticoid synthesis requires at least moderate inflammation and might be location dependent

The synthesis of corticosterone was induced in proximal colon by acute colitis, but it was not associated with increased expression of steroidogenic genes, *Lrh-1* and *Cyp11b1*. With the rationale that histopathological damage in DSS colitis increases towards distal colon (Perše and Cerar, 2012), corticosterone production was measured in distal colon in the study investigating enalapril and losartan. However, then DSS failed to induce corticosterone synthesis or gene expression above baseline. The results could be interpreted in two

different ways. Either the induction of colonic corticosterone synthesis requires at least moderate to severe inflammation, as the colitis was overall milder in the last study, or that corticosterone production is not uniform across colon, but rather takes place in certain segments of the intestine. The latter is supported by our previous results that corticosterone production is in fact much more prominent in the small intestine than in colon, and the level of synthesis decreases gradually from duodenum to ileum (Mattila et al., 2016).

In the experiments that glucocorticoid synthesis was induced (Studies I and II), *Lrh-1* and *Cyp11b1* mRNA were not increased by colitis. Activation of transcription factor LRH-1 is regulated by post translational modifications, like phosphorylation and ligand binding (Fernandez-Marcos et al., 2011), whereas steroidogenic enzymes are mainly regulated by transcription and availability of substrates (Hanukoglu, 1992). Induction of intestinal *Cyp11b1* has been reported alongside increased glucocorticoid synthesis in DSS and TNBS colitis studies in C57BL/6 mice (Ahmed et al., 2019; Coste et al., 2007; Noti et al., 2010a). Induction of *Lrh-1*, on the other hand, seems to require substantial provoking of the immune system with agents like LPS or T-cell activating antibodies (Mueller et al., 2006; Noti et al., 2010b). TNFα is a critical activating signal for intestinal glucocorticoid synthesis (Noti et al., 2010a), and its induction paralleled induction of glucocorticoid synthesis in Study II, but neither was induced in Study III. The choice of mouse strain is likely to affect intestinal glucocorticoid synthesis, as BALB/c mice are more resistant to DSS colitis than C57BL/6 mice, and their immunological response to colitis is polarized towards Th2 phenotype, instead of Th1 like in C57BL/6, leading to lower TNFα induction (Yang et al., 2017). Together this supports the assumption, that at least moderate induction of colitis is required to trigger intestinal glucocorticoid synthesis, as seen in the differences between our studies, and that the basal steroidogenic enzyme production is sufficient to increase glucocorticoid synthesis upon inflammatory stimuli without induction of *Cyp11b1*. However, since active glucocorticoids can be produced by 11β-HSD enzyme by conversion of 11-dehydrocorticosterone to

corticosterone in mice, an alternative synthesis pathway converting inactive adrenal-derived 11-dehydrocorticosterone to active corticosterone in colon cannot be ruled out based on our data.

6.3.2 Ang II induces glucocorticoid synthesis in jejunum

Ang II production is increased in colonic mucosa during experimental colitis (Khajah et al., 2016), and we wanted to test if Ang II participates in upregulation of intestinal corticosterone synthesis. Due to the limited size of mouse colon and the large amount of tissue needed, and because there are no indications that small and large intestine glucocorticoid synthesis would be differentially regulated (Bouguen et al., 2015a), the experiment was performed using small intestine samples. Pieces of mid jejunum were incubated in several concentrations of Ang II and the results show that Ang II can increase intestinal corticosterone synthesis *in vitro*. This has not been previously shown in the intestine, but Ang II has similar effects in adrenal *zona fasciculata* (McKenna et al., 1978; Parker et al., 1983; Spinedi et al., 1989). Regulation of adrenal and intestinal glucocorticoid synthesis are different in several ways. ACTH and steroidogenic factor-1 do not activate glucocorticoid synthesis in intestinal epithelium like they do in adrenals, which is instead in intestine under regulation of transcription factor LRH-1 (Coste et al., 2007). An intestine-specific peptide hormone which would increase glucocorticoid production has not been identified, but TNFα is required for intestinal glucocorticoid synthesis (Noti et al., 2010a), whereas it is a negative regulator of adrenal glucocorticoid synthesis (Jaattela et al., 1991; Villar et al., 2013). Our results suggest that intestine and adrenals, however, share similarities in Ang II-mediated induction of glucocorticoid synthesis. Upregulation of corticosterone by the generally proinflammatory Ang II suggests a feedback loop to regulate inflammation, and thus a new anti-inflammatory property for Ang II. Since it is established that Ang II induces TNFα in many cell types (Rosa et al., 2012; Sriramula and Francis, 2015), we propose a possible pathway

in which an inflammation-induced Ang II promotes glucocorticoid synthesis via TNFα in the intestine. Incidentally, parallel induction of colonic TNFα and corticosterone was observed in our studies, but the involvement of Ang II needs to be verified.

6.3.3 RAS inhibitors do not modulate glucocorticoid synthesis *in vitro* or *in vivo*.

As Ang II could induce glucocorticoid production in small intestine *in vitro*, we wanted to investigate the consequences of RAS inhibition on intestinal glucocorticoid synthesis. Basal glucocorticoid production could not be inhibited by captopril *in vitro*, implying that captopril does not affect the activity of LRH-1 or the steroidogenic enzymes in glucocorticoid synthesis pathway in healthy intestine. We next investigated whether RAS inhibitors could modulate colitis-induced glucocorticoid synthesis *in vivo*, and administered ACE inhibitors captopril and enalapril, angiotensin II receptor blocker losartan and ACE-inhibiting Ile-Pro-Pro in animals receiving DSS. Glucocorticoid synthesis was induced in the study using captopril and Ile-Pro-Pro, but while neither of the compounds modulated glucocorticoid production nor gene expression of *Lrh-1*, captopril virtually abolished *Cyp11b1* expression, offering some support to the involvement of RAS in regulation of intestinal glucocorticoid synthesis. There are no other reports of the effects of RAS inhibitors on intestinal steroidogenesis as far as we know, but some studies have evaluated their effects on adrenal corticosterone synthesis in rats. In one study, captopril had no effect on *Cyp11b1* expression in adrenals (LeHoux and Tremblay, 1992), but in other studies intracerebroventricular captopril administration reduced plasma corticosterone (Berecek et al., 1988), and ramipril and candesartan reduced plasma corticosterone, but only when stimulated with corticotropin-releasing hormone (Raasch et al., 2006). When we tested enalapril and losartan alone or in combination, they had no effect

on intestinal glucocorticoid synthesis or gene expression *in vivo*, but the lack of induction of glucocorticoid synthesis by DSS in that study is a major confounding factor in regard of comparison of the RAS inhibitors. Put together, inhibition of RAS does not seem to modulate the inflammation-induced corticosterone synthesis in the intestine, but our results indicate that RAS inhibitors might be different in regard of their ability to modify steroidogenic gene expression.

6.4 Future aspects and clinical relevance

As the pathogenesis of IBD involves both very general and very specific pathways, they are difficult and costly to treat with medications due to adverse effects and drug resistance. New treatment options are needed.

Due to the broad physiological and pathophysiological roles of intestinal RAS, the effects of RAS inhibitors on intestinal inflammation are pleiotropic and complex, and thus there is likely no simple explanation on the beneficial response to colitis. In part, it may be due to reduced proinflammatory factors, reduced motility and maintaining fluid homeostasis thus alleviating diarrhea, reduction in leukocyte adhesion, reduced scarring and control of apoptosis and proliferation (Bernstein et al., 2013; Fishlock and Gunn, 1970). To date, there is convincing evidence that RAS is involved both in human IBD and experimental colitis (Garg et al., 2015c; Hume et al., 2016; Jaszewski et al., 1990; Khajah et al., 2016; Shi et al., 2016), and that inhibiting RAS activation provides beneficial anti-inflammatory effects in experimental colitis (Jahovic et al., 2005; Liu et al., 2016; Spencer et al., 2007). The safety of RAS inhibitors has been demonstrated in treatment of cardiovascular diseases, they are inexpensive, readily available and lack the immunogenicity of new biologic medications, while affecting the same biological processes as current IBD medications (Hume and Radford-Smith, 2008). The feasibility and efficacy of

ACE inhibition or angiotensin II receptor blockade as a treatment for human IBD still needs to be investigated.

MSCs are advanced therapy medicinal products, which are emerging as a new kind of treatment for IBD. Cell-based treatments are complex both in their function and manufacturing, and slow to emerge in general use. The first MSC product (Panes et al., 2016), Alofisel™, by TiGenix and Takeda has been approved for marketing in 2018 for treatment of complex perianal fistulas in Crohn's disease in Europe, but none has yet been approved for luminal disease. Several trials are ongoing according to ClinicalTrials.gov.

The studies in this thesis are all preclinical in nature, and their results encourage to continue investigations of RAS inhibitors and MSCs in the treatment of intestinal inflammation. They provide new information of RAS in intestinal inflammation, specifically of the induction of ACE shedding by colitis. Correlation of inflammation severity and ACE shedding and comparison of the phenomenon in different experimental colitis and *in vitro* models would clarify the pathophysiological regulation of ACE shedding. We found evidence that RAS participates in the regulation of glucocorticoid synthesis in intestine, which should be verified in colon and *in vivo*, for example by measuring mucosal Ang II levels or in Ang II infused animals, and prompts lucrative questions of the potential interactions of the anti-inflammatory glucocorticoids and proinflammatory classical RAS in intestinal inflammation.

7 CONCLUSIONS

This series of studies investigated new potential treatments for intestinal inflammation using inhibitors of renin-angiotensin system, and mesenchymal stromal cells, analyzed changes brought by colitis in intestinal renin-angiotensin system and glucocorticoid synthesis, and examined their interactions particularly in a setting of intestinal inflammation. The conclusions based on the key findings of this thesis are:

- Inhibition of renin-angiotensin system by enalapril or losartan alleviates DSS-induced colitis non-synergistically by reducing inflammation in the colon, encouraging investigations of RAS inhibition in clinical studies of human inflammatory bowel diseases.
- Platelet-lysate expanded mesenchymal stromal cells are feasible in the treatment for intestinal inflammation, but although efficacy of treatment was modest in a xenogeneic model, they too have potential to be studied in clinical setting.
- These studies revealed, for the first time, a specific induction of ACE shedding by intestinal inflammation, which paralleled the severity of colitis based on more prominent shedding in distal colon, the most affected location in DSS-colitis, and by increased DSS concentration. The ACE-inhibiting peptide, Ile-Pro-Pro, and mesenchymal stromal cells reduced ACE protein expression and shedding, which all together implies that cell surface proinflammatory ACE is actively regulated by shedding of the protein.
- Ang II was found to induce intestinal corticosterone synthesis *in vitro*, whereas inhibition of ACE or angiotensin II receptor blockade did not affect glucocorticoid synthesis *in vitro* or *in vivo*. This implies that RAS activity participates in regulation of intestinal glucocorticoid production, thus revealing an indirect anti-inflammatory property of Ang II. We suggest the mechanism of Ang II upregulating

glucocorticoid synthesis would be via TNFα, which is a known inducer of intestinal glucocorticoid synthesis.

8 ACKNOWLEDGEMENTS

These studies were carried out at the Department of Pharmacology, Faculty of Medicine, University of Helsinki during 2014-2018. They were financially supported by Juhani Aho Foundation for Medical Research (formerly Finnish Clinical Chemistry Research Foundation) and Finska Läkaresällskapet, Einar och Karin Stroems stiftelse.

I am truly grateful to my supervisors Professor Riitta Korpela and Professor Emeritus Heikki Vapaatalo for their endless guidance and encouragement, which kept the goal always feel within reach and left no room for doubt. Riitta's empowering guidance gave me freedom grow as a scientist, and I appreciate it greatly. Heikki's vast experience in science and tireless counseling and interest in me and my project are inspiring and have carried me forward even in tough times.

I am grateful to the reviewers of my thesis, Docent Annika Meinander and Docent Riina Nieminen, whose insightful and encouraging comments helped to improve my thesis. Warm thanks go to Dr. Ewen MacDonald for the language revisions of the manuscripts.

I want to express my gratitude to all co-authors and collaborators during this project. My special thanks to Dr. Jere Lindén, whose expertise in pathology and interest in the project were insightful and truly invaluable. I sincerely thank Dr. Johanna Nystedt, Dr. Anita Laitinen and Dr. Matti Korhonen for the collaboration in the MSC study. Johanna's high standards drove me to improve myself and Anita's friendly attitude made work with the project easy. Without the help and meticulous work of Mervi Holappa during my maternity leave, I could not have finished my project in the time I did.

My warmest gratitude to my co-workers of all the hilarious time at the lab and the office A227b. Sharing the office with the great company of Aino Siltari, Richard Forsgård, Veera Kainulainen and Tuomas Heini has been a privilege

and a driving force throughout the project. Special thanks to Richard Forsgård for the insider-info of the group when I was looking for a PhD project and for introducing me to the lab. Thanks to Anne Kivimäki, Hanna Ventola, Sanni Lindroos and Liisa Lehtoranta for the brief time we shared in the group. I owe my deepest gratitude to all the students Lauri Pasanen, Hanna Launonen, Tomi Issakainen, Alessandro Accardi and Ninni Mattila. You taught me more than I could teach to you.

I wish to thank all the staff and colleagues for making the department a good and a welcoming place. The help and support of Sari Laakkonen at the beginning of my project is truly appreciated. I have greatly enjoyed the fun conversations with Päivi Leinikka, whose help and advice has been invaluable. My thanks to Anneli von Behr, who showed me around when I first started and Nada Bechara and Lahja Eurajoki, who remain as the backbones of the lab. Also, thanks to Piet Finckenberg, Viljami Jokinen, Oleg Kambur, Milla Lampinen, Teemu Aitta-Aho and Heidi Hytönen for all the conversations and Docent Petri Hyytiä for good tips for writing.

My friends and family have been the greatest support and for that and everything else I am truly grateful. My dear parents, Mali and Rami, have provided ear, shoulder, hugs and childcare as needed, and encouraged me to follow my dreams and ambitions in a sensible cocktail. My sister Taru who together with a sister-in-spirit, Nina Katajavuori, showed example of life in academia. My sister, Tea, and Eddy, and their crazy company throughout the years. The toughest woman I know, Tyyne-mummu, whe instilled her resilience on us all. The most grateful to I am to my husband, the love of my life, Uffe, whose incredible support has driven me forward, and my most loved children, Ronja and Aada, who taught me not to let work get in the way of life, nor life get in the way of dreams.

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